EVALUATION OF INTESTINAL RESPONSES TO ALTERNATIVE PROTEIN SOURCES FOR RAINBOW TROUT *(Oncorhynchus mykiss)*

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ABSTRACT

Replacement of fish meal as the primary protein source in diets for farmed carnivorous fish is a major priority for sustainability of the aquaculture industry. Three plant-based protein sources (soybeans, field peas, and canola) were investigated to compare their effects on the health and performance of rainbow trout (*Oncorhynchus mykiss*) and to identify significant anti-nutritional factors (ANFs). Six separate 8-week studies were conducted, over a period of one year, to assess the effects of protein source and processing level (meal versus protein concentrate) at dietary inclusion rates of 0 to 300 g kg^{-1}.

Abundance of inflammatory and immune marker transcripts including proliferating cell nuclear antigen (PCNA), immunoglobulin M (IgM), interleukin-1 *beta* (IL-1β), interleukin-8 (IL-8), and interleukin-10 (IL-10) was evaluated in distal intestinal tissue by quantitative PCR (qPCR) analysis. Activity of the pro-apoptotic enzyme caspase-3 was also assayed in distal intestinal tissue. Transcript abundance was highly variable and no suitable genes for the internal normalization of qPCR data could be identified. As a result, transcript copy numbers were reported per 50 ng of total RNA. At 300 g kg^{-1} inclusion, soybean meal (SBM) increased abundance of IL-8 and IgM, pea meal (PM) increased abundance of IL-10, and canola protein concentrate (CPC) increased abundance of IL-8. Pea protein concentrate (PPC) reduced IL-8 abundance and caspase-3 activity, while increasing abundance of IL-10. Canola meal (CM) and soy protein concentrate (SPC) did not significantly affect the transcript abundance of any assayed gene. Pearson correlation coefficients were determined between gene transcript abundance, performance parameters, protein source, inclusion level, and ANF content. Specific growth rate (SGR) was negatively correlated with the abundance of IL-1β and IgM. Dietary inclusion of SBM was positively correlated with all assayed proinflammatory markers and negatively correlated with SGR. Inclusion of PM was positively correlated with both SGR and the abundance of IL-10. The inclusion of CM was negatively correlated with average daily feed intake (ADFI) and with the abundance of both IL-8 and PCNA. Inclusion of PPC correlated positively with SGR and negatively with the activity of caspase-3. Correlation between transcript abundance and dietary content of putative ANFs suggested negative correlations between glucosinolate content, proinflammatory cytokine expression, SGR, and ADFI; whereas,

isoflavone content was positively correlated with proinflammatory markers and negatively correlated with SGR.

In conclusion, although high SBM and CM inclusion levels have been associated with reduced growth performance in trout, only SBM was associated with increased abundance of inflammatory marker transcripts. These contrasting responses may be mediated by CM glucosinolates, which could negatively affect palatability without inducing a pro-inflammatory response. Dietary PM was very well tolerated and may have promoted anti-inflammatory activity. Further processing of protein meals to concentrates markedly reduced any observable negative impact on performance parameters and the abundance of inflammatory marker mRNA transcripts. Interestingly, both PM and PPC were positively correlated with SGR and may contain a beneficial anti-inflammatory component.

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LIST OF ABBREVIATIONS

ADFI	-	Average daily feed intake
ANF	-	Antinutritional factor
bp	-	Base pair
cDNA	-	Complementary deoxyribonucleic acid
CGM	-	Corn gluten meal
CLR	-	C-type lectin receptor
CM	-	Canola meal
CPC	-	Canola protein concentrate
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxyribonucleotide triphosphate
dsRNA	-	Double-stranded ribonucleic acid
EF-1α	-	Elongation factor 1-*alpha*
IEL	-	Intraepithelial lymphocyte
IFN	-	Interferon
IgM	-	Immunoglobulin M
IL	-	Interleukin
IP5	-	Inositol pentakisphosphate
IP6	-	Inositol hexakisphosphate
LPS	-	Lipopolysaccharide
MAC	-	Membrane attack complex
MAMP	-	Microbe-associated molecular pattern
MBM	-	Meat and bone meal

MHC	-	Major histocompatibility complex
mRNA	-	Messenger ribonucleic acid
NF-κB	-	Nuclear factor *kappa*-light-chain-enhancer of activated B cells
NSP	-	Non-starch polysaccharides
PCNA	-	Proliferating cell nuclear antigen
PCR	-	Polymerase chain reaction
PM	-	Pea meal
PMN	-	Polymorphonuclear leukocyte
PPC	-	Pea protein concentrate
PRR	-	Pattern recognition receptor
qPCR	-	Quantitative real-time polymerase chain reaction
RNA	-	Ribonucleic acid
rRNA	-	Ribosomal ribonucleic acid
SBM	-	Soybean meal
SGR	-	Specific growth rate
SPC	-	Soy protein concentrate
Taq	-	*Thermus aquaticus*
TLR	-	Toll-like receptor
TNF-α	-	Tumour necrosis factor-*alpha*

1.0 INTRODUCTION

Annual global demand for food fish was estimated to be 136.2 mmt or a per capita equivalent of approximately 19.2 kg, as of 2012. This is a level which is unprecedented and has experienced steady growth over the past 50 years (FAO, 2014). Apparent per capita consumption of fish has increased significantly from a level of 9.9 kg yr^{-1} in the 1960's or approximately 33.0 mmt annually (FAO, 2014). In addition to an increase in the world population of approximately 4.1 billion from 1960 to 2012, the apparent per capita consumption of fish also increased by 93.9% placing a huge demand on industry (FAO, 2014). Some estimates predict that this demand could reach levels as high as 270.9 mmt by the year 2050, based on an increase in per capita consumption to approximately 30.4 kg yr^{-1} and a projected world population of 8.9 - 9.4 billion (Wijkström, 2003; PRB, 2009). Increases in demand can be largely attributed to the steadily increasing needs of a growing world population and to the changing diets of increasingly health conscious consumers in developed countries. If the outlined projections are accurate, a major challenge exists for the current supply system to meet future demand. In order to accomplish this, food fish will have to be supplied by both capture fisheries and the aquaculture industry.

The state of world capture fisheries is a subject of significant debate in both scientific and political circles; however, the general consensus is that production levels have either peaked or passed their peak in most regions. In the future we can expect to see maintenance of current production levels in well managed fisheries and likely an overall decline of production in the industry as a whole (FAO, 2014). As a result, capture fisheries are not currently in a position to increase long-term production and the onus will be on the aquaculture industry to meet growing consumer demand.

It is clear that significant and sustained growth of the aquaculture industry is required but major constraints exist. The production of feeds for the aquaculture industry is currently dependent on supplies of both fish meal and fish oil provided by capture fisheries. As a result of the aforementioned state of world fisheries, there is a finite supply of these commodities. This restriction establishes a limit to expansion of the industry in its current state. Total fish meal and fish oil production levels, averaged from 2010-2012, were 6.10 and 0.98 mmt respectively, down from highs of 7.48 and 1.50 mmt in 1994 (Tacon *et al.*, 2011 FAO, 2014). Annual production levels can fluctuate significantly based on several factors, including: capture levels of key

species, environmental impacts, fisheries management, *et cetera*. The Peruvian anchoveta (*Engraulis ringens*), which is of particular importance to fish meal and fish oil production, has the highest production levels of any single species but can also be highly variable, with annual capture rates of 4.2 - 8.3 mmt from 2008 - 2012 (FAO, 2014). According to FAO (2014), these large fluctuations are directly attributable to effects of the El Niño Southern Oscillation in the Southeast Pacific Ocean, where the anchoveta is primarily harvested. Despite these annual fluctuations, production levels of both fish meal and fish oil have been steadily declining since approximately 2005 due to decreased output and tighter regulation of fisheries (FAO, 2014).

Conversely, the consumption of fish meal and fish oil by the aquaculture industry has steadily increased, until recent years. In 1995, approximately 1.87 mmt of fish meal and 0.46 mmt of fish oil were utilized in the production of aquafeeds. This equated to approximately 27.3% and 33.6% of total production values for that year. By 2007, 3.84 mmt of fish meal and 0.82 mmt of fish oil, about 68.4% and 81.3% of total annual production, were used (Tacon *et al.*, 2011; FAO, 2014). New and Wijkström (2002) estimated that by the year 2015 the aquaculture industry would be responsible for consumption of approximately 68.0% of fish meal and 100.0% of fish oil produced worldwide, leaving little room for expansion. These predictions, though well founded at the time, have not quite come to fruition. Due to widespread knowledge of the existing challenges, research and implementation of improved feeding strategies and dietary formulations has resulted in a significant decrease in the use of fish meal and fish oil, despite increased production levels. By 2010, only 3.67 mmt of fish meal and 0.76 mmt of fish oil were used, down from their respective highs of 4.23 mmt in 2005 and 0.82 mmt in 2007 (Tacon *et al.*, 2011). Current predictions estimate that fish meal usage will continue to decline to approximately 3.49 mmt per annum and that fish oil usage will slowly increase to approximately 0.91 mmt by the year 2020 (Tacon *et al.*, 2011). Steady declines in fish meal usage will be achieved through reduction in inclusion levels by supplementation with a range of alternative protein sources, supported by ongoing research. Increased usage of fish oil is predicted due to the current lack of a suitable and cost-effective replacement, containing adequate levels of bioavailable highly unsaturated fatty acids (HUFAs). Research is ongoing in this field, with potential contributions through the use of marine microalgae, bacteria, or oils from genetically modified plants, but these technologies have not yet reached maturity (Tacon *et al.*, 2011; Zhang, 2013; FAO, 2014). Furthermore, in some species, research suggests that it may be possible to

completely remove marine-based ingredients from the diet. In the case of fish oil, partial or complete replacement with an alternative lipid source requires that the necessary enzymatic mechanisms to elongate and desaturate short-chain fatty acids, allowing for *de novo* production of eicosapentaenoic acid (C20:5 ω-3, EPA) and docosahexaenoic acid (22:6 ω-3, DHA), are present. While some species do possess these mechanisms and are able to survive and successfully reproduce in the absence of marine-based ingredients, conversion efficiencies are generally poor and the levels of EPA and DHA present in the final product are normally lower than in wild or traditionally fed fish (Zhang, 2013; FAO, 2014; Lazzarotto *et al.*, 2015).

The concentration of contaminants in farmed fish has also been of some concern in recent years. A study by Hites *et al.* (2004) found that the level of organochlorine compounds observed in farmed salmon was significantly higher than that in wild salmon. The concentrating effect of consumption of these compounds, present in both fish meal and fish oil, was determined to be the cause of these results (Hites *et al.*, 2004). Based on these observations, it is evident that a reduction in the inclusion of both fish meal and fish oil in the diets of farmed fish would have two major benefits. Firstly, it would result in a more efficient usage of these relatively scarce commodities, thereby extending the upper limit for growth of the industry; secondly, reduced intake of organochlorine compounds could result in decreased levels of these contaminants in farmed fish, providing a healthier product to the consumer and increasing social license of the aquaculture industry in the marketplace. These observations are particularly significant for carnivorous species, such as Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), since their intake of fish meal is proportionally much higher than other farmed species.

An opportunity exists to support the increased replacement of fish meal in the diets of such species with alternative sources of high-quality protein. Research into plant-based alternative protein sources is not a novel concept, but significant hurdles still exist to achieving high inclusion levels. Plant-based ingredients normally contain many compounds, deemed antinutritional factors (ANFs), which can have a negative impact on the intestinal health and overall performance of fish (Collins *et al*., 2013). This is especially true for carnivorous species, since they are not well adapted to the intake of plant-based ingredients and are therefore prone to a host of negative impacts.

The bulk of research conducted to date has been focused on the inclusion of soybean meal (SBM) in the diets of both Atlantic salmon and rainbow trout. Baeverfjord and Krogdahl

(1996) showed an association between dietary SBM inclusion and a condition termed soybean meal induced enteritis, identified in Atlantic salmon. Subsequent studies have confirmed these findings and expanded our understanding of this condition. It has been determined that SBM inclusion levels as low as 20% can result in pathology of the intestine, characterised by: increased cell proliferation in the distal gut, increased width of the lamina propria, infiltration of immune cells into the intestinal mucosa, decreased supranuclear vacuolization of enterocytes, and decreased length of microvilli. These changes in physiology are most prominently observed in the distal intestine, reducing absorptive capacity and nutrient utilization, resulting in decreased growth performance and feed efficiency. (Burrells *et al.*, 1999; Bakke-McKellep *et al.*, 2000; Buttle *et al.*, 2001; Krogdahl *et al.*, 2003; Sanden *et al.*, 2005).

Though the effects of increasing SBM concentrations in the diet are well established, a detailed understanding of the factors and processes mediating these effects has yet to be determined. Investigation of these areas could provide insight to the factors limiting inclusion of SBM in salmonid diets and aid in the development of strategies to safely increase usage. Plant-based alternative protein sources, other than SBM, have not been thoroughly examined. Canola and pea-based protein sources show promise as potential candidates due to their high protein content and desirable amino acid balance. Several studies have been conducted investigating the feeding effects of such ingredients in salmonids; however, the focus has been on digestibility and growth performance factors (Higgs *et al.*, 1995; Stickney *et al.*, 1996; Thiessen *et al.*, 2003 & 2004; Drew *et al.*, 2005 & 2007; Øverland *et al.*, 2009; Alami-Durante *et al.*, 2010; Gao *et al.*, 2011). The prospect therefore exists for further research into canola and pea-based protein sources, in terms of establishing their impact on intestinal physiology and the limitations of dietary inclusion.

Additionally, the drive to maximize growth performance and feed efficiency with inclusion of novel ingredients has often come at the expense of animal welfare. Feeding diets which directly or indirectly result in intestinal inflammation and diarrhea is not an acceptable practice, whether or not it results in decreased performance. A focus on the health and welfare of animals is vital to all investigations in this area. The identification of ingredients and feeding strategies which are beneficial not only to producers and to the industry as a whole, but are also favourable to the animals in our care should be the ultimate goal.

2.0 LITERATURE REVIEW

2.1 Taxonomic Classification of Rainbow Trout

The rainbow trout can be broadly classified as a carnivorous teleost. Teleostei is the largest and most diverse of the three infraclasses of the class Actinopterygii or ray-finned fishes, along with Holostei and Chondrostei, and includes nearly all species of sport and commercial fish worldwide. In fact, Teleostei is the largest and most diverse biological group of all vertebrates, including 44 orders, and encompassing more than 30,000 species, roughly equivalent to all other vertebrate groups combined. In all, teleosts represent about 95% of all known fish species and cover a massive range of types and sizes, from eels to anchovies and from minnows to marlins (Near *et al.*, 2012). Rainbow trout can be more specifically classified as a member of the family Salmonidae, commonly referred to as salmonids, and the subfamily Salmoninae, which they share with several other species of trout, salmon, and charr. Atlantic salmon are members of this subfamily and the subject of a significant body of research in the area of plant-based alternative protein sources.

Historically, the taxonomy and phylogeny of species has been largely based on fossil records and the interpretation of distinguishable morphological characteristics. More recently, advances in the fields of genetics and molecular biology have contributed significantly to the employed methodologies and ultimately resulted in the emergence of the field of evolutionary genomics. The use of modern analytical methods, such as sequence-based analysis of mitochondrial DNA, has resulted in an enhanced understanding of the speciation of this group and the factors influencing their evolution. One such factor, which is thought to be an important driver of eukaryote evolution, is the phenomenon of polyploidy or whole-genome duplication. The incidence of polyploidy and its significance in the diversification of species have not been thoroughly investigated in animals; however, it is known to be common in plants and is thought to be a significant source of evolutionary pressure (McGrath and Lynch, 2012).

Salmonids are thought to have undergone at least four separate genome duplication events in their evolutionary history. Currently, salmonids are considered to be tetraploid and are undergoing the process diploidization through the progressive loss and inactivation of duplicated genes (Braasch and Postlethwait, 2012; Near *et al.*, 2012; Berthelot *et al.*, 2014; Glasauer and Neuhauss, 2014). Berthelot *et al.* (2014) estimate that the ancestral genome, prior to the salmonid-specific fourth whole-genome duplication event (Ss4R), which occurred approximately

96 Mya (±5.5 Mya), contained approximately 31,500 genes. Currently, the rainbow trout genome contains 46,585 protein-coding genes of which approximately 48% are retained as duplicates. While this level of genomic analysis is well beyond the scope of this project, it is important to consider when investigating the incidence of specific genes and potential influences on transcript abundance.

2.2 Intestinal Physiology of Rainbow Trout

2.2.1 Gross Intestinal Anatomy and Mucosal Morphology

In nature, the diet of the rainbow trout consists mainly of plankton, insect larva, insects, snails, clams, and other fish (Teixeira and Cortes, 2006). The size of food that the animal consumes tends to increase proportionally with body size. Carnivores tend to exhibit a shorter intestinal length, in relation to body size, than herbivores due to the relative ease of digestion and absorption of their diet (Ray and Ringø, 2014). This is due to the fact that less retention time and less epithelial surface area are required to achieve efficient digestion and absorption of nutrients. By the same logic, the passage rate of digesta through the gut of a carnivore tends to be faster than in herbivores (Ray and Ringø, 2014). Rainbow trout have a relatively short gastrointestinal tract, with a body length to gut length ratio in the range of 5:6, a feature common to carnivorous teleosts (Burnstock, 1959; Ezeasor, 1978). These points are of particular significance, since the inclusion of plant ingredients in the diet inherently decreases digestibility and ease of processing in comparison to the natural diet or standard commercial aquaculture diets for rainbow trout. The fact that the digestive system is so well adapted to their natural diet presents challenges to the use of plant ingredients in aquafeeds and is the primary cause of negative impacts that can be associated.

The mouth is characterized by a large gape and sharp, pointed teeth common to carnivores. The esophagus is relatively short and contains a thick, striated circular muscle coat serving the purpose of moving ingesta from the pharynx to the stomach, a distance of only a few centimeters (Burnstock, 1959; Ray and Ringø, 2014). Mucosal folds, composed of columnar epithelium, dominate the esophageal lining (Burnstock, 1959). Mucus-secreting cells are also present in the epithelium, which provide lubrication to aid in the passage of ingesta and help to protect the esophageal lining from damage (Clements and Raubenheimer, 2006). The tissue of

the esophagus is also very elastic in nature, an adaptation in predatory fish to allow for the passage of relatively large prey items, such as other fish, which are consumed whole (Clements and Raubenheimer, 2006).

The esophagus opens into the large cardiac stomach, which contains both circular (inner) and longitudinal (outer) smooth muscle, in addition to submucosa, muscularis mucosa, stratum compactum, and stratum granulosum layers which are absent in the esophagus (Burnstock, 1959; Ray and Ringø, 2014). The cardiac stomach continues into the pyloric stomach, which is the site of preliminary acidic and proteolytic digestion; as well as the mechanical breakdown of ingesta. This region possesses a circular muscle layer approximately five times the thickness of that found in both the cardiac stomach and the following intestine (Burnstock, 1959; Ray and Ringø, 2014). The gastric epithelium contains several cell types, including: oxynticopeptic cells which secrete both HCl and pepsin; enteroendocrine cells which secrete hormones such as gastrin and somatostatin; and mucous-secreting cells which provide protection for the stomach against both mechanical damage and the low pH of gastric secretions (Clements and Raubenheimer, 2006).

The proximal intestine contains numerous diverticula, called the pyloric cecae, which are unique to fish and serve to greatly increase the surface area and therefore absorptive capacity of this region without increasing the overall length or thickness of the intestine (Burnstock, 1959, Clements and Raubenheimer, 2006; Ray and Ringø, 2014). Bergot *et al.* (1975) estimated that the combined length of the pyloric cecae in rainbow trout is greater than six times that of the intestine. They also estimated the combined surface area of this region to be more than double that of the intestine (Bergot *et al.*, 1975). Interestingly, these structures are seen to be more developed in carnivorous species than in herbivores, particularly those with a short overall gut length (Clements and Raubenheimer, 2006).

The structure of the mid-gut is similar to that of the cardiac stomach, except for the absence of the submucosa and muscularis mucosa layers (Burnstock, 1959). The muscular coats are relatively thinner than those found in the stomach and the mucosa is organized into thin longitudinal folds, or villi, supported by a slim core of connective tissue (Weinreb and Bilstad, 1955). In addition, the stratum compactum and stratum granulosum layers are quite prominent in this region (Burnstock, 1959).

The rectal or distal gut region differs quite prominently in its organization from that of the mid-gut. The muscular coat in this region is more powerful than that of the mid-gut, being

approximately twice the thickness. More strikingly, the annulospiral septa or ileorectal valves present in this area dominate the landscape, protruding into the lumen of the rectum (Burnstock, 1959). These structures serve to increase the surface area of the distal gut, increase retention time, and likely prevent the backflow of digesta (Burnstock, 1959; Ezeasor and Stokoe, 1980).

The pancreas and liver are also important components of the digestive system, secreting several essential substances into the intestine. The pancreas is not a distinct organ in most teleosts, including rainbow trout (Clements and Raubenheimer, 2006). It instead consists of dispersed nodules throughout the adipose tissue associated with the pyloric cecae (Branson, 1993; Clements and Raubenheimer, 2006). The pancreas serves a dual role, with both endocrine and exocrine functions. The exocrine component is that which secretes digestive enzymes into the intestinal tract. These enzymes include: trypsin, chymotrypsin, elastase, collagenase, α-amylase (pancreatic amylase), and carboxypeptidases; all of which are important for the breakdown of ingested foods (Clements and Raubenheimer, 2006). Exocrine pancreatic secretions also contain a significant concentration of bicarbonate ions (HCO_3^-). These bicarbonate secretions function to neutralize acidic chyme entering the intestine from the stomach and establish a neutral to slightly basic pH of ~7.3 in the proximal intestine, allowing for optimal activity of digestive enzymes (Saghari Fard *et al.*, 2007).

The liver is also a very important organ in the digestive process. Though it has many other vital functions in the body, hepatic contributions are absolutely essential to the efficient digestion and absorption of lipids. The liver is responsible for the production of bile, which contains bile acids synthesized from cholesterol. Hepatic secretions are delivered to the digestive tract via the hepatic duct (or bile duct), which intersects with the intestinal lumen just distal to the pyloric sphincter, at the beginning of the proximal intestine (Burnstock, 1959; Leitritz, 1959). In rainbow trout, the primary bile acids produced are cholic acid and chenodeoxycholic acid, respectively accounting for approximately 85% and 14% of total production (Denton *et al.* 1974). These steroid acids are amphipathic in nature and are essential for the emulsification of lipids in digesta, through formation of micelles. The distribution of lipid droplets into micelles greatly increases the effective surface area for enzymatic action and allows for their efficient digestion and absorption (Bakke *et al.*, 2011; Ray and Ringø, 2014).

2.2.2 Intestinal Epithelial Cell Function and Replacement

The intestinal epithelium is a versatile system and performs several functions necessary for the digestion and absorption of nutrients, as well as immune and barrier functions. The predominant cell types in the intestinal epithelium of rainbow trout are simple columnar enterocytes and goblet cells (Yamamoto, 1966). Goblet cells are named for their goblet-like shape; they produce and maintain a protective layer of mucus through the synthesis and secretion of high-molecular weight glycoproteins known as mucins (Specian and Oliver, 1991). The enterocytes are long and narrow, approximately 70 µm in length and 10 µm in width, with microvilli 2-3 µm in length and 0.1 µm in diameter (Yamamoto, 1966; Sire *et al.*, 1981). Enterocytes are the absorptive cells of the intestine and have several functions including the uptake of water, electrolytes, sugars, peptides, amino acids, lipids, and unconjugated bile salts from the intestinal lumen (Bakke *et al.*, 2011; Ray and Ringø, 2014). The apical surface of the intestinal epithelium also contains a number of membrane-bound enzymes, essential for the complete hydrolysis of carbohydrates and proteins, which are adsorbed in the glycocalyx of enterocytes (Kuz'mina and Golovanova 2004). These membrane-bound enzymes are also known as brush border enzymes, some examples for rainbow trout include: alkaline phosphatase, leucine aminopeptidase, maltase, isomaltase, lactase, and sucrase (Krogdahl *et al.*, 2003; Krogdahl *et al.*, 2004). Whether lactase activity is associated with the brush border or other cell structures is of some debate (Krogdahl *et al.*, 1999). The joint action of these cells serves as a selective barrier between the intestinal lumen and the body, allowing the passage of nutrients and the exclusion of unwanted or potentially harmful entities. Enteroendocrine cells are also present in the intestinal epithelium. This cell type accounts for less than one percent of all epithelial cells but is responsible for the secretion of substances such as cholecystokinin, vasoactive intestinal peptide, and substance P, which are essential for the endocrine regulation of the digestive system (Ezeasor and Stokoe, 1981; Holmgren *et al.*, 1982).

The dynamics of cell replacement in the intestinal epithelium are congruent with that of mammals, though rainbow trout do not possess a true crypt (Vickers 1962; Ezeasor and Stokoe, 1981). New cells are continuously generated in a proliferative zone, commonly located at the base of the villi. Undifferentiated cells are produced in this region and migrate towards the tip of the villus, propelled by continued cell proliferation in their wake. During their migration, cells differentiate to a specific type and take on their role in the epithelium. These cells continue to

migrate until they are either sloughed off at the tip of the villus, undergo the process of apoptosis, or become necrotic due to physical damage or some other insult (Vickers 1962; Ezeasor and Stokoe, 1981). The rates at which these activities occur may be influenced by many factors including nutritional status, diet, environmental factors, and stress.

2.3 Nutrient Digestion and Absorption in Trout

This section will examine the detailed processes involved in the digestion and absorption of dietary components in the digestive tract of rainbow trout. The three major nutritional components of the diet (carbohydrates, proteins, and lipids) will be examined separately, since the mechanisms involved in their digestion and absorption differ. The fourth section will examine vitamins, minerals, and electrolytes. The roles of mechanical breakdown, enzymatic digestion, the intestinal brush border, and nutrient transport will be examined in each of the following sections. Digestibility ranges differ for different ingredients and dietary components. By definition, any material which is not broken down or is not absorbed in the gut is indigestible (FAO, 1980).

2.3.1 Carbohydrates

The digestion of dietary carbohydrates primarily occurs in the proximal intestine and pyloric cecae. Prior to reaching the intestine, low gastric pH also contributes to carbohydrate digestion, aiding in denaturation and providing less restricted access for digestive enzymes to do their work (Clements and Raubenheimer, 2006). Several enzymes are commonly involved in the breakdown of carbohydrates, including: pancreatic amylase (α-amylase), lactase, and the brush border enzymes maltase, isomaltase, and sucrase (FAO, 1980; Krogdahl *et al.*, 2003; Krogdahl *et al.*, 2004). The action of two or more enzymes is normally required to achieve complete hydrolysis of ingested carbohydrates (Clements and Raubenheimer, 2006).

Alpha-amylase hydrolyses starches by cleaving α-1,4-glycosidic bonds, producing disaccharides and trisaccharides, including: maltose, isomaltase, maltotriose, and dextrins. In addition to these products, ingested molecules of sucrose, lactose, and maltose are further acted on by the aforementioned brush border enzymes before they can be absorbed by the intestinal epithelium (Bakke *et al.*, 2011; Ray and Ringø, 2014). Following the complete hydrolysis of

these carbohydrates to their component monosaccharides, absorption occurs across the intestinal epithelium by either facilitated diffusion or secondary active transport depending on the monosaccharide in question. Glucose and galactose are transported into enterocytes by Na^+-dependent symporters, such as sodium/glucose co-transporter 1 (SGLT1) (Coady *et al.*, 1990; Pajor *et al.*, 1992). The monosaccharides fructose, mannose, xylose, and arabinose may also be present. The absorption mechanisms for these sugars have not been definitively established in rainbow trout (FAO, 1980). After entering an enterocyte, monosaccharides pass into the circulation of the villus by facilitated diffusion (FAO, 1980; Bakke *et al.*, 2011; Ray and Ringø, 2014).

Generally speaking, carnivorous teleosts are not known for their ability to efficiently utilize high levels of carbohydrates in their diet (Wilson, 1994; Moon, 2001). Though the processes of carbohydrate digestion and absorption do not significantly differ between carnivorous, herbivorous, and omnivorous species, there are observable differences in both the levels and regulation of digestive enzymes and transport mechanisms. Significantly lower levels of enzymes such as α-amylase and maltase are seen in carnivores, as compared to herbivorous or omnivorous species, as well as decreased numbers of glucose transporters essential for the absorption of digestion products from the intestinal lumen (Buddington *et al.*, 1997; Hidalgo *et al.*, 1999; Krogdahl *et al.*, 2005). Buddington and Hilton (1987) found that rainbow trout apparently lack the ability to regulate maltase expression in response to diet, showing no change in enzyme activity between diets containing or lacking carbohydrates. Rainbow trout have also been observed to be incapable of regulating the level of intestinal glucose transport in response to dietary changes (Buddington, 1987). These deficiencies in the ability of rainbow trout to effectively digest and absorb carbohydrates, in comparison to herbivorous or omnivorous species, are no doubt an adaptation to their naturally protein-rich diet, but are of particular interest and concern when modifying aquaculture feeds to include increasing levels of plant-based ingredients.

2.3.2 Proteins

The digestion of proteins begins in the stomach, where denaturation by the low pH of gastric secretions occurs. Loss of tertiary structure reduces steric hindrance, allowing digestive enzymes physical access and enabling them to cut proteins into smaller fragments, called peptides. Three

proteolytic enzymes are responsible for this initial digestion: pepsin is secreted in the stomach; trypsin and chymotrypsin are secreted in the proximal intestine by the pancreas. These enzymes are secreted in an inactive form, called zymogens, in order to protect the body from auto-digestion. Pepsinogen is cleaved to pepsin by HCl in gastric secretions. Trypsinogen is cleaved to trypsin by enterokinase, an enzyme produced by specialized cells of the intestinal epithelium. Once activated, trypsin can then cleave both trypsinogen and chymotrypsinogen to liberate more trypsin and chymotrypsin in the proximal intestine. Carboxypeptidase and elastase are also present in the proximal intestine, secreted by the pancreas, and aid in the breakdown of proteins into smaller peptides (FAO, 1980; Clements and Raubenheimer, 2006; Bakke *et al.*, 2011; Ray and Ringø, 2014).

These enzymes all share the action of proteolysis, but act in different ways. Pepsin, trypsin, chymotrypsin, and elastase are endopeptidases and work by cleaving bonds between specific pairs of non-terminal amino acids. For example, trypsin strictly cuts peptide bonds at sites directly after an arginine or lysine residue, unless followed by proline. Carboxypeptidase is an exopeptidase and acts by cleaving single amino acids from the carboxy-terminus (C-terminus) of peptides (FAO, 1980; Clements and Raubenheimer, 2006; Bakke *et al.*, 2011; Ray and Ringø, 2014).

The next step involves the further breakdown of the peptides produced by the previous proteolytic action, prior to absorption. The remaining peptides are acted on by two enzymes produced by cells of the intestinal microvilli or brush border. These brush border enzymes are aminopeptidase and dipeptidase. Aminopeptidase is an exopeptidase, much like carboxypeptidase, which acts by cleaving single amino acids from the amino-terminus (N-terminus) of peptides. Dipeptidase is also defined as an exopeptidase, since it technically cleaves a terminal amino acid, though its true action is to cleave dipeptides, liberating the final two amino acids from a peptide (FAO, 1980; Clements and Raubenheimer, 2006; Bakke *et al.*, 2011; Ray and Ringø, 2014).

Regional differentiation of the intestine gives rise to two primary modes of protein absorption (Sire *et al.*, 1992). In the proximal intestine and pyloric ceca, protein absorption occurs by conventional means, very similar to that of mammals. Following hydrolysis, free amino acids, dipeptides, and tripeptides are absorbed by enterocytes primarily via Na^+-dependent secondary active transport mechanisms (Collie and Ferraris, 1995; Santigosa *et al.*, 2011). After

entering an enterocyte, di- and tripeptides are hydrolyzed to single amino acids and pass into the circulation of the villus by diffusion (Ingham and Arme, 1977; FAO, 1980; Bakke *et al.*, 2011; Ray and Ringø, 2014). In contrast to mammals, active transport mechanisms are present along the entire length of the intestine and differences in transporter substrate specificity exist (Collie and Ferraris, 1995; Buddington *et al.*, 1997; Santigosa *et al.*, 2011).

Specialized absorption also occurs in the distal gut, where it has been observed that trout have the capacity to absorb protein macromolecules by the process of pinocytosis (Ezeasor and Stokoe, 1981; Georgopoulou *et al.*, 1985, 1986). This process of macromolecule absorption and intracellular digestion is actually present in all teleosts, during the larval stage, but is generally lost later in development, for species with a true stomach, when gastric secretions and luminal protein digestion commence (Ezeasor and Stokoe, 1981; Rombout *et al.*, 1985). This is not the case in rainbow trout, where distinct supranuclear vacuolization of enterocytes and the apparent ability of these cells to absorb and digest protein macromolecules are retained into adulthood (Ezeasor and Stokoe, 1981). The reasons for and functionality of this system are not entirely clear.

Based on the morphology and biochemistry of the gut, rainbow trout are fully capable of complete intraluminal digestion of proteins, posing an interesting problem for explaining the retention of this larval adaptation (Smit, 1968; Ezeasor, 1978). Ezeasor and Stokoe (1981) proposed two plausible explanations for this occurrence. The first is that, as an adaptation to their high-protein diet, the retention of an intracellular method of protein digestion serves as a backup to primary digestion, ensuring the maximum utilization of ingested protein. The second possibility proposed by Ezeasor and Stokoe (1981) relates to the structure of the gastrointestinal tract and its correlation with diet. The wall of the stomach in rainbow trout is relatively inelastic due to the presence of the stratum compactum, a continuous tissue layer present in the wall of the stomach and intestine. The stratum compactum is made up of collagen fibres which provide stability and rigidity to the gut wall, but also render it relatively stiff and limit its storage capacity due to the inability to significantly distend when filled (Weinreb and Bilstad, 1955; Burnstock, 1959; Ezeasor and Stokoe, 1981). Ezeasor and Stokoe (1981) proposed that in the presence of an abundant food source, the rate of gastric emptying could be increased due to an increase in food intake and lack of storage capacity. A decrease in gastric retention time could have a significant impact on the degree to which ingested proteins are denatured, hydrolyzed, and absorbed by the

intraluminal route. In this situation, the existence of a secondary means of protein acquisition, in the form of pinocytosis and intracellular digestion of protein macromolecules, would complement the primary route and increase the overall efficiency of protein digestion and absorption (Ezeasor and Stokoe, 1981).

2.3.3 Lipids

Lipid digestion and absorption in rainbow trout does not differ fundamentally from the process in higher vertebrates. The most common source of lipids in the diet is triglycerides, an ester of three fatty acids bound to a glycerol backbone. The hydrolysis of triglycerides must occur before they can be absorbed by the intestinal epithelium.

The breakdown of dietary triglycerides likely begins in the stomach. Esterase activity has been documented in this region and the possibility of other lipase involvement cannot be ruled out (FAO, 1980). Despite the occurrence of any gastric action, the primary sites of lipid digestion are the proximal intestine and pyloric cecae, where the complete hydrolysis of ingested triglycerides occurs (Ostos Garrido *et al.*, 1993). In mammals, pancreatic lipase is specific to the hydrolysis of fatty acid moieties occupying positions one and three of triglycerides. The action of this enzyme produces two free fatty acids and a β-monoglyceride (Bakke *et al.*, 2011; Ray and Ringø, 2014). The rarity of pinocytotic vesicles in enterocytes of the proximal intestine and pyloric cecae supports the suggestion that triglycerides are completely hydrolyzed in the lumen and that the absorption of mono- and diglycerides by this route does not occur (Ostos Garrido *et al.*, 1993). Complete luminal hydrolysis of triglycerides indicates either that rainbow trout pancreatic lipase does not display the specificity seen in mammals (Patton *et al.*, 1975) or the activity of a β-monoglyceride lipase is responsible for the completion of triglyceride hydrolysis (Léger and Bauchart, 1972).

Absorption of dietary lipids is accomplished by enterocytes in the proximal intestine and pyloric cecae (Ostos Garrido *et al.*, 1993). Short-chain fatty acids, with a length of less than twelve carbons, are generally capable of passing into enterocytes by simple diffusion (Bauermeister *et al.*, 1979; Bakke *et al.*, 2011; Ray and Ringø, 2014). The products of triglyceride hydrolysis which cannot pass directly into enterocytes, including long-chain fatty acids such as eicosapentaenoic acid (C20:5 ω-3, EPA) and docosahexaenoic acid (22:6 ω-3, DHA), are ferried to the apical surface of enterocytes in the form of micelles, in combination

with bile salts secreted by the liver. Delivery of these products, in high concentration, directly to the apical membrane, allows for their diffusion into enterocytes while bile salts remain in the lumen and are recycled for continued usage. Once inside an enterocyte, the majority of fatty acids are re-esterified with glycerol into triglycerides (Higgins and Barnett, 1971). The newly formed triglycerides are then transported out of enterocytes and into the lamina propria in the form of chylomicrons or very low-density lipoproteins (VLDLs) (Bauermeister *et al.*, 1979; Sire *et al.*, 1981; Vernier, 1990). Bauermeister *et al.* (1979) also suggested that a portion of dietary lipids, likely those short-chain fatty acids capable of passing into enterocytes by simple diffusion, may be transported directly across the intestinal epithelium and into the blood stream in the form of free fatty acids.

2.4 Innate Immunity and Intestinal Inflammation in Salmonids

This section will examine the immune system of salmonids, as it relates to the gastrointestinal tract. Discussion will be limited to the components of the innate immune system present in the intestine and to the inflammatory response in the gut. These processes will be discussed in detail, including factors influencing their activation and their impacts on the gut and overall health. These areas are of key importance for understanding the impact that novel feed ingredients can have on the gut and the animal as a whole.

The hallmarks of innate immunity, in contrast with acquired or adaptive immunity, are: antigen-independent response, non-specific modes of action, no associated immunological memory, and an immediate maximal response to activation. This type of immune protection is an ancient evolutionary adaptation and is found in all classes of plants and animals, many of which do not benefit from any form of adaptive immunity (Vasta and Lambris, 2002; Flajnik and Du Pasquier, 2004; Foey and Picchietti, 2014). In many ways, the immune system of teleost fish is akin to that found in higher vertebrates, including mammals. As a result of their developmental environment, devoid of maternal shelter and defences, developing fish are forced to defend themselves even from an embryonic stage. This situation forces them to rely heavily, if not exclusively, on non-specific defence mechanisms. Not surprisingly, teleosts have very well developed innate immune systems, on par with those found in mammals (Ellis, 2001; Magnadóttir, 2006; Uribe *et al.*, 2011; Foey and Picchietti, 2014). Mature teleosts also have a significant level of adaptive immunity. In fact, members of the class Osteichthyes or bony fish,

to which the teleosts belong, are some of the most primitive creatures to possess the type immunoglobulin and T-cell based adaptive immunity commonly found in higher classes of vertebrates (Flajnik *et al.*, 1999; Whyte, 2007). Though not as diverse as that found in mammals, the adaptive immune response in teleost fish is relatively advanced and a significant part of the overall immune repertoire of these animals.

The protective measures of the innate immune system fall broadly into one of four categories: anatomical and biological barriers, humoral barriers, cellular mechanisms, and inflammatory response (Magnadóttir, 2006; Whyte, 2007; Foey and Picchietti, 2014).

2.4.1 Anatomical and Biological Barriers

Anatomical barriers exist at all levels of innate immunity; some examples include skin, mucus, acids, enzymes, and commensal microorganisms. These systems confer protection to the organism by physically, chemically, and biologically preventing the attachment, intrusion, or survival of antigens and pathogens. These systems can also include mechanical factors such as mucociliary clearance in terrestrial animals or peristaltic action in the intestinal tract, working to clear foreign bodies from the airway and preventing settling or antigen attachment in the gut, respectively (Paustian Roberts, 2012). Where anatomical barriers are concerned, the gastrointestinal tract itself is a physical, chemical, and biological barrier between an organism and the outside world.

Beginning in the mouth and continuing through the entire tract, in varied form, the cells of the esophageal, gastric, and intestinal epithelia act as a primary physical barrier, directly preventing the entry of unwanted or potentially harmful entities, while selectively allowing or facilitating the passage of others. So long as they are intact, the tight junctions between epithelial cells create an effective barrier which is essentially impermeable to most unwanted molecules or organisms. Secretions such as saliva and mucus also play an important role. Saliva is not present in fish but mucus is essential for lubrication and protection of the gastrointestinal tract, preventing physical damage to the epithelium in addition to other functions (Magnadóttir, 2006; Whyte, 2007; Uribe *et al.*, 2011; Foey and Picchietti, 2014). Specifically, mucus is a viscous secretion from goblet cells of the epithelium. It consists mainly of high-molecular weight glycoproteins, known as mucins, which are responsible for its viscosity and functionally contribute to lubrication and barrier functions. Some mucins contain transmembrane domains

which anchor them to the apical surface of the epithelium. In addition to their protective role, these membrane-bound mucins can also act as receptors for signal transduction. Protection from some organisms can be achieved by blocking physical access to the intestinal epithelium, thereby preventing adhesion and colonization. Alternatively, membrane-bound mucins may act as detachable traps for organisms which are capable of binding with them directly. Upon binding, the luminal portion of the protein can be released from its transmembrane anchor and carry the organism away, effectively preventing adhesion to the epithelium (Lindén *et al.*, 2009). Mucus can also contain other components such as secreted immunoglobulins and enzymes, like lysozyme, which provide antimicrobial properties (Magnadóttir, 2006; Whyte, 2007; Uribe *et al.*, 2011; Foey and Picchietti, 2014).

In the stomach, the relatively low pH of gastric acid (~3.0), combined with a minimum residency time of four to eight hours, is an effective chemical barrier to many pathogens (Bucking and Wood, 2006; Sugiura *et al.* 2006; Yúfera *et al.* 2012). Unless they are somehow protected or capable of survival in an acidic environment, gastric acid secretions are generally effective at limiting both the type and volume of viable microorganisms capable of passing through to the intestine. It is important to note that, unlike mammals, salmonids only passively acidify the stomach, in response to food intake. During periods of fasting, a relatively neutral pH is maintained. To my knowledge, the effect of this passive acidification on immunity or gut inflammation has not been investigated; however, it is conceivable that the volume of viable microorganisms reaching the intestine could be increased during periods of fasting (Sugiura *et al.* 2006; Yúfera *et al.* 2012).

Upon exiting the stomach, constant movement of digesta and mucus through the digestive tract is facilitated by peristaltic contractions of the intestinal smooth muscle layers. This action keeps the chyme in near-constant motion, helping to reduce attachment of organisms to the epithelium and allowing for clearance of organisms and antigens from the system. Additionally, mucin secretions from goblet cells create a secondary physical barrier by maintaining a continuous unstirred layer at the boundary of the intestinal epithelium, which all compounds or organisms must pass through in order to access the apical surface of an enterocyte (Magnadóttir, 2006; Whyte, 2007; Uribe *et al.*, 2011; Foey and Picchietti, 2014).

Bile acids and digestive enzymes secreted into the lumen act as potent protective agents, in addition to their roles in the digestive process. These compounds are capable of disrupting

bacterial membranes and hydrolyzing viral and bacterial proteins, helping to limit the proliferation of organisms in the gut. More specifically targeted antimicrobial compounds, such as defensins and cathelicidins, are also present. Antimicrobial peptides such as these are an important part of the innate immune system, having the capability to defend against a broad range of potential pathogens, including: bacteria, fungi, viruses, and parasites (Yang *et al.*, 2004; Groot *et al.*, 2006; Collet, 2013; Foey and Picchietti, 2014). Defensins are a class of small (<50 amino acids) cationic peptides. Their cationic properties allow them to interact with negatively charged molecules, such as lipopolysaccharides (LPS) found in the cell membrane of Gram-negative bacteria, lipoteichoic acid (LPA) found in the cell wall of Gram-positive bacteria, or negatively charged glycoproteins found in the envelope of some viruses. It is by this interaction that most defensins function, creating pores and compromising the integrity of membranes. Through this same affinity, defensins are also capable of binding to free LPS and neutralizing its potentially harmful effects. Defensins are defined by a β-sheet structure bound by three disulfide bonds. They are divided into two main classes; *alpha* and *beta*, based mainly on their size and the pattern of disulfide bonding present (Ganz, 2003). A third class, *theta*-defensins, are extremely rare and have only been identified in a handful of species. Prior to their secretion, defensins exist in an inactive form, called prodefensins, stored within intracellular granules. Activation occurs when these prodefensins are secreted and undergo enzymatic cleavage, freeing the active peptide (Ganz, 2003). To date, four novel *beta*-like defensins (omDB-1, omDB-2, omDB-3, and omDB-4) have been identified in teleost fish and are thought to be important for their antiviral properties and their effect on the ability of organisms to colonize epithelial surfaces (Falco *et al.*, 2008; Casadei *et al.*, 2009; Collet, 2013). No *alpha*-like defensins have yet been identified in teleost fish. Defensins can be produced by immune cells, such as neutrophils, and by almost all epithelial cells, including enterocytes (White *et al.*, 1995). Many are produced continuously, but production can also be stimulated or increased in response to signalling. In higher vertebrates, the presence of proinflammatory cytokines or microbe-associated molecular patterns (MAMPs) is known to stimulate increased production of these peptides (Powers and Hancock, 2003). These signalling pathways are moderated by pattern recognition receptor (PRR) detection of specific ligands, such as LPS, flagellin, or dsRNA. Unfortunately, the ligand specificity of PRRs in rainbow trout has not yet been well defined, making the factors influencing regulation of these pathways unclear (see section 2.3.4).

Cathelicidins, much like defensins, are small (<100 amino acids) cationic peptides produced by immune cells, such as neutrophils or macrophages, and by epithelial cells (Gennaro and Zanetti, 2000; Nizet and Gallo, 2003; Foey and Picchietti, 2014). Many of the behaviours and antimicrobial properties of cathelicidins are akin to defensins, due to the antimicrobial specificity derived from their cationic state. Defensins and cathelicidins are distinguished mainly by their structural characteristics and evolutionary origins. While the structure of defensins is fairly homogenous, cathelicidins are highly diverse, having significant variability in their structure and a wide range of sizes. The hallmark of this class of peptides is that, in their inactive form, they are all stored intracellularly and bound to a generally highly conserved N-terminal sequence, known as the cathelin domain. This sequence is so named due to its high similarity to a cysteine proteinase inhibitor of the same name, originally isolated from porcine leukocytes. When stimulated to do so, the inactive peptides are presented at the cell surface and released in their diverse active forms through enzymatic cleavage, typically by neutrophil elastase, from the cathelin domain prosequence (Gennaro and Zanetti, 2000; Zanetti, 2004). To date, two novel cathelicidin genes have been identified in rainbow trout, designated *rt*CATH_1 and *rt*CATH_2 (Chang *et al.*, 2005; Chang *et al.*, 2006). Chang *et al.* (2006) also identified two additional cathelicidin genes in Atlantic salmon, designated *as*CATH_1 and *as*CATH_2. It is likely that these genes and the peptides that they code for play an important role in the innate immune system of these teleost fish. When considering the significance and impact of these genes, it should also be considered that these, and other genes found in pairs, are likely examples of the estimated 22,000 genes that are retained as ohnologues from the most recent (Ss4R) whole-genome duplication event (Berthelot *et al.*, 2014).

In addition to their role in innate immune function, there is also evidence that both defensin and cathelicidin antimicrobial peptides are involved in the stimulation and signalling of an adaptive immune response. Both defensins and cathelicidins have been found to be chemotactic to a wide range of immune cells in mammals. Human β-defensins are chemotactic to immature dendritic cells and to memory T-cells (Yang *et al.*, 1999). Human cathelicidin is chemotactic to T-cells and to phagocytes, including neutrophils and monocytes (Agerberth *et al.*, 2000; Yang *et al.*, 2000). It is not known, at this time, whether these chemotactic properties exist for the β-defensins and cathelicidins of teleost fish but it is not unreasonable to suspect that they do. Overall, the known antimicrobial and protective effects of these peptides, combined with

their likely role in the attraction of a more specialized immune response, indicate that these compounds presumably play a significant role in the regulation of microbial growth, colonization, and pathogen elimination in the intestine of teleost fish (Foey and Picchietti, 2014).

In healthy fish, a significant biological barrier to infection exists in the form of a diverse and stable population of microorganisms present in the gut, mainly comprised of bacteria but also containing fungi and other microorganisms. Through competitive exclusion, the commensal microbiota forms a substantial obstacle to most pathogens. The established population is diversified and occupies all physical, chemical, and nutritional niches available for colonization. For rainbow trout, the dominant bacterial species which make-up the intestinal microbiota are those belonging to the phyla Firmicutes and Proteobacteria (Mansfield *et al.*, 2010; Desai *et al.*, 2012). It was found that bacterial species belonging to these phyla account for more than 90% of detectable sequences, with species belonging to the phyla Actinobacteria and Bacteroidetes comprising the majority of the remainder (Mansfield *et al.*, 2010; Desai *et al.*, 2012). Competitive exclusion works to protect the host from infection by making it very difficult for invading organisms to thrive or even to gain a foothold. In order to be successful, a potential pathogen must essentially overcome or subvert an entrenched and well-fortified army on their home turf. The capacity of the commensal microbiota to exclude other organisms, including pathogens, is mediated by a number of mechanisms. Significant competition exists for essential nutrients, with a clear advantage going to the resident population which is stable and well-adapted to the environment. The commensal organisms occupy physical space, limiting opportunities for other organisms to colonize, especially where epithelial attachment / association is required. Finally, commensal microbiota can also directly protect their niche from invaders through the production of antimicrobial, bacteriostatic, and bactericidal compounds such as bacteriocins. These are a diverse class of proteinaceous toxins, produced by most bacteria, mainly targeted at limiting the growth and proliferation of other closely related species. These toxins generally have a narrow spectrum of specificity, though there are some notable exceptions which have a broad-spectrum of activity. One of these broad-spectrum bacteriocins, called nisin, is produced by the Gram-positive bacterium *Lactococcus lactis* and is effective against a wide range of Gram-negative organisms, as well as spores. There has been interest and investigation into the use of this and other bacteriocins as feed additives for commercial aquaculture production, intended to limit the proliferation of specific pathogenic organisms, such

as *Listeria monocytogenes* and *Escherichia coli*, and to improve the shelf-life of products through activity against organisms such as lactic acid bacteria (Bakkal *et al.*, 1999).

2.4.2 Humoral Barriers

Humoral barriers are also an active and essential part of the innate immune system. These systems are defined by their association with components of the blood and other bodily fluids, historically referred to as humors. Humoralism is a disproven medical theory, originating from ancient Greek and Roman science, which proposed that the body was made up of four distinct fluids, known as humors. The four humors were blood, black bile, yellow bile, and phlegm. It was believed that illness occurred when these humors were not in the correct balance (Hart, 2001). Though this ancient theory is now known to be false, the word 'humor' survives as a term for bodily fluids. Some examples of systems involved with humoral immunity include the complement system, the coagulation system, and iron sequestration systems.

The complement system is an essential part of innate immunity, so-named because it complements and enhances both the activity and effectiveness of antibody-mediated and cellular mechanisms for pathogen clearance. The main functions of the complement system are to opsonize antigens making them more susceptible to phagocytosis, to attract immune cells to antigens through chemotaxis, to lyse foreign cells by rupturing their plasma membranes, and to cause clumping of antigens making them less mobile and easier to attack. The advanced mammalian complement system is a collection of more than 30 serum globular proteins, some of which are soluble and some cell-bound. Although more primitive, containing approximately 21 homologous components, the complement system of teleost fish is a critical element of their immune system (Claire *et al.*, 2002; Boshra *et al.*, 2006; Nakao *et al.*, 2011; Foey and Picchietti, 2014). These proteins exist mainly in an inactive form, requiring proteolytic cleavage to become active. Much like the digestive enzymes present in pancreatic secretions, these systems have the potential to do serious harm to host tissues and must therefore be tightly regulated and controlled. Complement proteins are activated by specific mechanisms and respond in a sequential manner, referred to as the complement cascade. Activation can take place by three main pathways: the classical pathway, the alternative pathway, and the lectin pathway.

The classical pathway is activated when the complement protein designated C1 is bound by an appropriate antigen-antibody complex, involving an antibody such as immunoglobulin M

(IgM) (Claire *et al.*, 2002; Boshra *et al.*, 2006; Nakao *et al.*, 2011). The next step is the activation of components C2 and C4 which, when cleaved, recombine to form the enzyme C3 convertase, from C2a and C4b, resulting in the hydrolysis of component C3 to C3a and C3b. The remaining components, C2b and C4a help to enhance the local inflammatory response by acting as chemoattractants for immune cells such as neutrophils. The alternative pathway is constantly active at a low level but kept in check by control proteins present in the blood. This low-level activity is due to the fact that complement component C3 is inherently unstable in an aqueous environment and spontaneously cleaves into components C3a and C3b. If these products are not quickly activated, they are destroyed by control proteins such as complement Factor H. Control proteins such as these are constantly present in the blood, at levels higher than the complement components that they control, keeping the cascade suppressed. Full activation of the alternative pathway occurs when an antigen is bound by complement component C3b. The lectin pathway is initiated when mannose-binding lectin (MBL) forms a complex with an antigen. Mannose-binding lectin forms complexes with specific types of sugar residues found on the surface of many pathogenic bacteria, fungi, and some viruses. This pathway proceeds in a manner analogous to the classical pathway, with the MBL-antigen complex resulting in the activation of components C2 and C4 (Claire *et al.*, 2002; Boshra *et al.*, 2006; Nakao *et al.*, 2011).

Once the complement cascade has been activated by any of these pathways it proceeds spontaneously, resulting in opsonisation of antigens by C3b, enhancement of inflammation by C3a, C4a, and C5a, and formation of the membrane attack complex (MAC), which creates pores in the plasma membrane of target cells, initiated by C5b. With respect to activation and enhancement of the inflammatory response, C3a, C4a, and C5a are all classified as anaphylotoxins capable of stimulating mast cell and phagocyte degranulation resulting in a localized inflammatory response. These components also stimulate chemotaxis of mast cells and other immune cells, particularly neutrophils. The control proteins are responsible for bringing the cascade back under control once the level of antigen has decreased and there is no longer enough activation to overcome their negative feedback. The host is also more directly protected by other control proteins, such CD59 or protectin. This protein is present on host cell membranes and is responsible for preventing insertion of the MAC, thereby protecting host cells from lysis (Claire *et al.*, 2002; Boshra *et al.*, 2006; Nakao *et al.*, 2011).

The next humoral barrier for discussion is the coagulation system, the mechanism by which blood forms clots. Though it is not intrinsically part of the innate immune system, it does have some overlap in function and can contribute to overall immune function. This system, similar to the complement system, is comprised of many coagulation factors, mainly serine protease enzymes, which are constantly present in the blood in an inactive form. Activation of the system can occur by one of two pathways. The tissue factor or extrinsic pathway is the primary pathway for coagulation. Tissue factor, also known as coagulation factor III or thrombokinase, is a glycoprotein produced by cells which are not normally in contact with flowing blood, such as smooth muscle cells or fibroblasts. When exposed to flowing blood, normally due to trauma, tissue factor comes into contact with coagulation factor VIIa, forming an activated complex. The contact activation or intrinsic pathway is triggered by contact of circulating platelets with collagen, underlying endothelial tissue, normally due to physical damage to blood vessels. This contact causes the platelets to bind directly with collagen, resulting in platelet activation. Following activation of either the extrinsic or intrinsic pathway the coagulation cascade proceeds, with each step catalyzing the next step in the reaction. The end result of the coagulation cascade is the formation of a stabilized cross-linked fibrin clot (Hanumanthaiah *et al.*, 2002; Tavares-Dias and Oliveira, 2009).

The process of coagulation is clearly vital for survival but is not fundamentally an immune process. Contributions of this system to innate immunity are largely incidental. Increased vascular permeability and chemotactic properties conveyed by some coagulation products contribute to the efficiency and targeting of phagocytic cells, which will be covered in the next section. Invading organisms can also be trapped by clot formation or directly assaulted by factors such as *beta*-lysine, which is an amino acid produced by platelets. *Beta*-lysine has innate immune activity because it acts as a cationic detergent, disrupting lipid barriers. It is capable of directly causing the lysis of many Gram-positive bacteria (Mayer, 2011).

In addition to the complement and coagulation cascades, a number of other humoral factors also contribute to the function of the innate immune system. Iron binding, transport, and sequestration are very important aspects of innate immunity. The proteins ferritin and transferrin are two examples of such systems which are present in teleosts (Tange *et al.*, 1997). Ferritin is an intracellular globular protein and transferrin is a glycoprotein present in blood plasma. Both are responsible for binding of ferric iron (Fe^{3+}) which is essential for the survival and proliferation of

invading organisms. An essential component of life, iron is required for many metabolic processes such as respiration and DNA synthesis. The action of these iron-sequestering proteins maintains a low level of free iron(III) in circulation, making it difficult for invading organisms to gain a foothold. As a result, many bacteria and fungi have evolved powerful mechanisms, such as siderophores, for attracting and binding the iron that they require for survival (Howard, 1999; Nevitt and Thiele, 2011).

Lysozymes and interferons are also important humoral factors, contributing essential antibacterial and antiviral activities respectively to the arsenal of the innate immune system. Lysozymes, also known as N-acetylmuramide glycanhydrolases, are glycoside hydrolase enzymes with potent bactericidal properties. Lysozymes destroy bacteria, particularly Gram-positive bacteria, by attacking peptidoglycans in their cell wall and ultimately causing cell lysis. Lysozyme is found in secretions such as mucus, as well as being present in serum, secreted by immune cells such as macrophages and polymorphonuclear leukocytes (PMNs) (Jollès, 1996; Rieger and Barreda, 2011; Collet, 2013).

Interferons are an important humoral immune component due to their function in preventing intercellular proliferation of viral infections and combating intracellular bacterial infections. Interferons are cytokines which are produced and secreted by host cells in response to the presence of a pathogen. So far, two interferon (IFN) classes have been definitively identified in fish, type I IFNs and interferon-*gamma* (IFN-γ), or type II IFN (Robertsen, 2006; Collet, 2013; Foey and Picchietti, 2014). Type I IFNs are responsible for promoting an antiviral state in host cells, helping to protect them from infection and thereby limiting viral proliferation. These IFNs are produced by host cells when they become infected. Infected cells recognize viral dsRNA via TLRs and react by secreting type I IFNs (Robertsen, 2006; Collet, 2013). Neighbouring cells have receptors for type I IFNs and are thereby alerted to the presence of the virus. Uninfected cells can then respond to this threat by producing antiviral proteins which help to protect them from infection when new virions are eventually released from the neighbouring infected cell (Robertsen, 2006; Collet, 2013).

Type II IFN or IFN-γ is the second interferon which has been identified in teleost fish. In fact, recent studies suggest that teleost fish have two types of IFN-γ, designated IFN-γ1 and IFN-γ2, a feature that is not present in humans (Purcell *et al.*, 2009). It is possible that this is another example of ohnologues retained from the most recent (Ss4R) whole-genome duplication event

(Berthelot *et al.*, 2014). Unlike the type I IFNs, which are expressed by all cells, IFN-γ is only produced by specialized immune cells such as nonspecific cytotoxic cells (NCCs), also called natural killer-like (NK-like) cells, in response to stimulation by interleukin 12 (IL-12) and interleukin 18 (IL-18) produced by phagocytes (Harris *et al.*, 1991; Biron and Sen, 2001; Zimmerman *et al.*, 2004). As part of the adaptive immune response, IFN-γ is also produced by other cell types such as $CD4^+$ T helper 1 (Th1) lymphocytes and $CD8^+$ cytotoxic T lymphocytes (CTL) (Robertsen, 2006). IFN-γ has many antiviral and immune regulating functions, including upregulation of major histocompatibility complex (MHC) class I molecules and antiviral proteins in most cell types. Particularly in macrophages and other antigen-presenting cells, activation is achieved through upregulation of several key genes by IFN-γ, including MHC class II molecules (Robertsen, 2006; Collet, 2013).

2.4.3 Cellular Mechanisms

Colloquially speaking, the specialized blood cells of the immune system are both the ground troops and the Special Forces in the war against infection. Nine specific cell-types are associated with the innate immune response, while others are exclusively related to acquired immunity, and some are involved in both. These cells not only perform the day-to-day duties of patrolling the body and removing potential hazards before they become a problem but are also responsible for responding to more immediate threats. The characteristics of teleost immune cells are highly analogous to their mammalian counterparts, which generally make a good basis for comparison where there is a lack of species-specific data available (Manning and Nakanishi, 1996; Whyte, 2007; Foey and Picchietti, 2014).

Broadly referred to as leukocytes, literally meaning white cell, this class of cell originates in the kidney and spleen. All members of this class differentiate from a single multipotent progenitor, called a hematopoietic stem cell (Catton, 1951; Miller *et al.*, 1998). It is worth noting that this is a significant departure from the mammalian system, where hematopoietic stem cell production occurs primarily in the bone marrow (Dorshkind, 1990). In a sense, leukocytes operate much like independent single-celled organisms within the host. They are not generally restricted in their movement and are basically on a search-and-destroy mission against any debris or foreign organisms that they encounter. These cells can operate independently or respond to chemical signals, calling them into action. The leukocytes associated with the innate immune

system include: NK-like cells, mast cells, eosinophils, basophils, neutrophils, monocytes, macrophages, dendritic cells, and γδ T cells (Ellis, 2001; Magnadóttir, 2006; Rieger and Barreda, 2011; Uribe *et al.*, 2011; Foey and Picchietti, 2014). Each of these cell types will be briefly described in the following section, outlining their basic functions and triggers.

Nonspecific cytotoxic cells (NCCs) or natural killer-like (NK-like) cells are cytotoxic lymphocytes, analogous to mammalian natural killer (NK) cells, belonging to a sub-set of leukocytes which also includes T cells and B cells. NK-like cells do not directly target antigens; their role is to detect and destroy infected host cells with the objective of limiting the spread of infection. This action is especially useful for management of viral infections and for tumour surveillance. Unlike other immune cells, which typically rely on antigen presentation for detection of infected cells, NK-like cells are able to detect stricken host cells without need for antibodies or MHC antigen presentation. This makes NK-like cells particularly efficient and able to respond more rapidly to viral infections than other cell types. Infected host cells are thought to be detected by NK-like cells through a state known as missing-self, which relies on recognition of a lack of self-identifying characteristics, namely the reduced expression of MHC class I molecules on the cell surface, rather than the presence of foreign characteristics (Moody *et al.*, 1985; Harris *et al.*, 1991; Zimmerman *et al.*, 2004).

Mast cells are resident cells found within connective tissues and mucous membranes throughout the body. These cells are associated with skin, nerves, blood vessels, lymphatic vessels, and any area that has close proximity or a direct interface with the outside environment. With respect to innate immunity, mast cells are involved in pathogen defence and wound healing. These cells are also associated with allergic reactions and anaphylaxis but these topics are not strictly relevant to this discussion. In general, mast cells function much like an alarm system for the body. They are strategically located in places which interface with the environment and have receptors on their surface to detect antigens which they come into contact with. Though not in direct contact with the lumen, they are commonly located just below the mucosal barrier and are capable of responding to luminal antigens, a process that is dependent on antigen sampling cells present in the epithelium (Fuglem, *et al.* 2010). Mast cells store a variety of bioactive compounds in intracellular structures called granules. These compounds include: histamine, heparin, proteoglycans, and serine proteases. These compounds are of varied class, containing inflammatory mediators, hormonal mediators, and chemoattractant cytokines or

chemokines. When activated, through binding of a recognized antigen to sites on the cell's surface, the stored compounds are released into the surrounding tissue in a process called degranulation. The release of these compounds has several pertinent effects on the body, primarily resulting in an immediate, localized inflammatory response due to the action of histamine. Increased permeability of surrounding vessels, recruitment of immune cells to the site, and ongoing production of cytokines and other mediators after degranulation also contribute to mast cell function. In addition to these primary functions, mast cells can also be classified as both phagocytes and antigen-presenting cells being capable of phagocytosis of bacteria and MHC class I presentation of antigens on their surface (Malaviya *et al.*, 1996; Malaviya and Abraham, 2001; Kalesnikoff and Galli, 2008).

Three individual cell-types make up a group called polymorphonuclear leukocytes (PMNs) or granulocytes, characterized by the presence of cytoplasmic granules and lobed nuclei of varied appearance. The individual cell-types which make up this group are neutrophils, eosinophils, and basophils (Ainsworth, 1992; Suzuki and Iida, 1992; Mayer, 2011; Rieger and Barreda, 2011). Neutrophils also happen to be professional phagocytes, meaning that phagocytosis is their primary function. They will be covered along with the other phagocytic cell-types, which will be discussed next.

Eosinophils are best known for their function in defence against multicellular parasites and other infections. Mature eosinophils circulate through the body and migrate to sites of infection, attracted by chemokines. The cytoplasmic granules of mature eosinophils contain compounds such as cytotoxins, antiviral ribonucleases, peroxidase, and neurotoxin. Degranulation occurs, releasing these compounds, when the cell comes into contact with an appropriate antigen, working directly to destroy it and to recruit reinforcements. Some of these compounds also stimulate other immune cells, resulting in increased mast cell degranulation, increased mucus production, and a host of other effects that contribute to the immune response. It was long-held that eosinophils were mainly involved in parasite and viral defence, but recent insights have painted a new picture. It is now clear that these cells serve a much broader purpose, acting to both initiate and disseminate inflammatory and immune responses to infection through production of a wide range of cytokines and other modulators (Ainsworth, 1992; Rothenberg and Hogan, 2006).

Basophils are in many ways like a circulating mast cell. The composition of their cytoplasmic granules and extra-cellular secretions are mirrored in mast cells, including: histamine, heparin, proteoglycans, proteolytic enzymes, and cytokines. These compounds contribute to inflammatory reactions, increased vascular permeability, and recruitment of other immune cells to the site of infection. They are particularly found at sites of allergic reaction or parasitic infection (Ainsworth, 1992).

The final broad classification that will be discussed is the phagocytes, meaning eating cells or devouring cells. This class of cells is responsible for protecting the host through the ingestion and destruction of foreign particles, microorganisms, and dead or dying host tissue (Mayer, 2011; Rieger and Barreda, 2011). Phagocytes work either by detecting targets via cell surface receptors or recruitment to the site of an infection by chemokines released from other cells. Once a target has been located, phagocytes begin engulfing them by wrapping a portion of their plasma membrane around and drawing the target inside. Once the target has been consumed, it is contained within a membrane-bound vesicle called a phagosome, generally protecting the phagocyte from being compromised. Within their cytoplasm, phagocytes also contain structures, called lysosomes, which hold a cocktail of around 50 acid hydrolase enzymes capable of hydrolyzing proteins, DNA, RNA, polysaccharides, and lipids. Within the lysosome, these enzymes are stored at their physiologically optimal pH of ~4.8, making them maximally potent upon release. Once the target is inside the phagocyte, lysosomes generally migrate to and fuse with the phagosome, releasing their contents and proceeding to digest the target (Secombes and Fletcher, 1992; Neumann *et al.*, 2001; Rieger and Barreda, 2011). The phagocytic cell types which will be covered include those which are considered to be professional phagocytes. This subdivision includes phagocytes for which the detection, capture, and destruction of harmful entities, such as bacteria or dead / dying host cells is their primary function. The cell-types that will be discussed are neutrophils, monocytes, macrophages, and dendritic cells.

Neutrophils, being also classified as PMNs, contain many of the same compounds in their cytoplasmic granules as the other members of this group; however, unlike eosinophils and basophils, approximately one third of neutrophilic granules are known as azurophilic granules, due to their histological staining characteristics. These azurophilic granules, also called primary granules, fuse with phagosomes when an antigen is consumed and contain a number of antimicrobial enzymes and defensins (Secombes and Fletcher, 1992; Neumann *et al.*, 2001;

Kumar and Sharma, 2010; Rieger and Barreda, 2011). Being highly motile, neutrophils are quickly attracted to sites of infection by the process of chemotaxis, responding to signals generated by other cells, such as interleukins or interferons. In addition to their role as both PMN and professional phagocyte, neutrophils exhibit another kind of duality. When circulating and inactive the cells are spherical, but once activated they change form. Activated neutrophils are amoeboid in appearance and behaviour. Their form is unstructured and they extend pseudopods while searching for and pursuing targets (Edwards, 1994).

In order for a neutrophil to recognize an antigen the target must normally be opsonized by antibodies or complement, so that the cell surface receptors of the neutrophil can identify it (Secombes and Fletcher, 1992; Neumann *et al.*, 2001; Kumar and Sharma, 2010; Rieger and Barreda, 2011). Once an antigen has been located, the neutrophil can respond in the same way as the other PMNs, undergoing degranulation and releasing a mixture of antimicrobial products into the surrounding area. In addition, the neutrophil can also respond by consuming and destroying antigens by the process of phagocytosis. Following phagocytosis, neutrophils generate a respiratory burst within the phagosome in addition to the binding of azurophilic granules. Respiratory burst, in no way related to respiration, is a common method used by phagocytes for intracellular destruction of antigens. Through secretion of specific enzymes and a series of reactions within the phagosome significant quantities of reactive oxygen species, such as the superoxide anion (O_2^-), are generated. This action results in the production of a range of substances, including hydrogen peroxide (H_2O_2) and hypochlorous acid (HClO), which have potent bactericidal properties (Secombes and Fletcher, 1992; Neumann *et al.*, 2001; Kumar and Sharma, 2010; Rieger and Barreda, 2011). Activated neutrophils also release a web of extracellular fibres called neutrophil extracellular traps (NETs). It is thought that these structures are used to trap and destroy antigens extracellularly, independent of phagocytosis (Palić *et al.*, 2007; Kumar and Sharma, 2010; Rieger and Barreda, 2011). Even if the neutrophil does not use NETs to destroy antigens, it is a useful strategy for containing their spread and allowing other immune cells a chance to destroy them.

Monocytes are specialized cells, capable of phagocytosis, which serve a dual purpose in the immune system. Mature monocytes can either be found circulating in the bloodstream or in storage in the spleen. They typically circulate for a relatively short period of time before migrating to the tissues of the body, where they change form. While circulating, monocytes

perform their initial function as active phagocytes, responding to chemical signals from sites of infection and generally patrolling the bloodstream for opsonized antigens that they may randomly encounter. Circulating monocytes also serve an important function as antigen-presenting cells. Using fragments derived from the destruction of phagocytized targets, the monocyte can present antigens through MHC molecules on their surface, triggering a more specific immune response. Following their migration into the tissues, monocytes differentiate, becoming resident macrophages or dendritic cells, a vital source of these cell-types in the organism (Secombes and Fletcher, 1992; Neumann *et al.*, 2001; Rieger and Barreda, 2011).

Macrophages, as mentioned above, are primarily derived from the stimulated differentiation of monocytes. They are professional phagocytes and are geared towards the attack of foreign bodies, damaged host cells, and invading microbes whether they are actively mobile or resident in a specific tissue location. Mobile macrophages function in essentially the same manner as activated neutrophils, having amoeboid characteristics and actively responding to stimuli from pathogens, chemokines, and damaged host cells. Resident macrophages are more focused on the removal of necrotic host tissue than mobile macrophages. They are located tactically, in locations where incursions are likely to occur, keeping the area clear of pathogens and recruiting additional immune responses as necessary. Macrophages also play an important role as antigen-presenting cells and as regulators of inflammatory and targeted immune responses through secretion of cytokines and other mediators (Secombes and Fletcher, 1992; Neumann *et al.*, 2001; Rieger and Barreda, 2011).

Dendritic cells are primarily professional antigen-presenting cells and act as an intermediary between the innate and adaptive immune systems. They serve this purpose by indiscriminately sampling and presenting a wide range of antigens, stimulating adaptive mechanisms, while also contributing to innate immune function through cytokine secretion and modulation of other classes of immune cells. These cells are so-named because at some stages of their development they possess branched projections, called dendrites, and bear a morphological resemblance to neuronal dendrites, though they are distinct in structure and function (Bassity and Clark, 2012). When present, these structures serve to greatly increase the surface area of the cell that is in direct contact with its surroundings, making it far more efficient at detecting antigens and cellular signals. Dendritic cells can be formed either by the stimulated differentiation of monocytes or by the maturation of immature specialized precursor cells through hematopoietic

activity in the kidney and spleen (Bassity and Clark, 2012). Cells formed from monocytes as well as those immature cells that have migrated to peripheral tissues are resident sentries in areas prone to incursion. Immature cells, produced in the kidney or spleen, are also found in circulation where they perform similar functions while migrating to peripheral tissues. Dendritic cells are continually sampling their surrounding environment and contain several triggering mechanisms, including TLRs and other PRRs, used for the detection of antigens (Rieger and Barreda, 2011; Bassity and Clark, 2012). Basic dendritic cell function involves sampling and processing of targets, presentation of antigens on MHC molecules, and migration to secondary lymphoid tissues where they interact with and activate other immune cells, such as T cells and B cells (Bassity and Clark, 2012). Unlike most other antigen-presenting cells, which are only capable of activating memory T cells, dendritic cells are also able to activate naïve T cells, allowing them to generate an immune response to previously unseen antigens and making them the most important of the antigen-presenting cells (Bassity and Clark, 2012). In addition to these roles, dendritic cells are also thought to play an important role in the prevention of autoimmunity through the sampling and presentation of self-antigens, resulting in the deletion or silencing of auto-reactive T cells and increased activity of regulatory systems (Steinman and Nussenzweig, 2002). Dendritic cells also act as important immune mediators through the production of cytokines. For example, interleukin 12 (IL-12) is produced in response to antigen stimulation and has a host of effects. These effects include stimulation of T cell growth, differentiation of T cells, and stimulation of cytokine production, such as IFN-γ and TNF-α, from other immune cells. A special class of dendritic cells, known as plasmacytoid dendritic cells, are also known to produce large amounts of type I interferons in response to TLR triggering (Workenhe *et al.*, 2010).

2.4.4 Pattern Recognition Receptors

Pattern recognition receptors (PRRs) are a class of specialized proteins that play a crucial role in surveillance, recruitment, and regulation of gene expression for the immune system. In general, PRRs are proteins expressed on the surface of immune cells and other cell types which are capable of recognising and binding to particular molecular patterns, such as those found in flagellin or LPS, which are commonly found in pathogens or other microbiota, but are distinct from the host. Many of these patterns are shared by large groups of potential pathogens, allowing relatively few receptor types to cover a huge number of targets. Non-host molecular patterns,

recognized by PRRs, are known as pathogen-associated molecular patterns (PAMP) or perhaps more appropriately, microbe-associated molecular patterns (MAMP), since they can be found associated with most microbes, not only those which are considered pathogenic. Pattern recognition receptors, being a broad classification, are subdivided into several families or classes, based on the types of molecular patterns that they are specific to and the affect that their activation has on the immune system. The classes of PRR found in teleost fish, to date, include C-type lectin receptors (CLR), NOD-like receptors (NLR) and, Toll-like receptors (TLR) (Zhang *et al.*, 2000; Zhang *et al.*, 2001; Rodriguez *et al.*, 2005; Palti *et al.*, 2006; Laing *et al.*, 2008; Palti, 2011; Foey and Picchietti, 2014).

C-type lectin receptors (CLR) are a collection of transmembrane proteins. They are grouped based on the presence of relatively conserved regions which are homologous to carbohydrate recognition sites, though they do not always bind such targets (Zelensky *et al.*, 2005; Geijtenbeek and Gringhuis, 2009). The CLRs are split into two groups in mammals. Group I are classified as mannose receptors and group II are asialoglycoprotein receptors. To date, no group I CLRs have been identified in teleost fish, while a handful of group II CLRs have been identified. Three group II CLRs have been identified in Atlantic salmon and two in rainbow trout. The specific ligands for these receptors and the extent of their contribution to the immune response are not fully understood but sequence similarity to mammalian homologues suggests that mannose-binding lectins, galactose, and other carbohydrates are likely candidates. Some of these CLRs have also been found to contain immunoreceptor tyrosine-based inhibition motifs (ITIMs), which are commonly associated with negative regulation of cell signalling (Vivier and Daëron, 1997; Zhang *et al.*, 2000; Zhang *et al.*, 2001; Soanes *et al.*, 2004).

Nucleotide-binding oligomerization domain-like receptors or NOD-like receptors (NLR) are a collection of proteins whose localization and specific function are not well defined. They are speculated to be cytoplasmic receptors localized within immune cells. Their role in the immune system is thought to be associated with detection of MAMPs within the cytoplasm of their host cell, perhaps related to phagocytosis or simply internal monitoring. There are around 20 NLRs in mammals divided into several subfamilies. Analysis of the zebrafish (*Danio rerio*) genome has shown that nearly all NLR-subfamily proteins are either conserved between humans and teleost fish or have teleost-specific analogues. It is believed that, among other things, these receptors are involved in signalling of caspases, involved in apoptosis pathways, as well as

regulation of inflammatory cytokine production through manipulation of NF-κB signalling. Due to the large number of conserved genes and representation across several investigated teleost genomes, it is expected that these receptors play an important role in the immune system of teleost fish (Franchi *et al.*, 2006; Laing *et al.*, 2008; Foey and Picchietti, 2014).

Toll-like receptors (TLR) are another family of transmembrane signalling proteins which are an important component of the immune response. There are between 10 and 15 TLRs identified in mammalian species. Many are highly conserved, while others have altered functions or are inactive in differing animals. As in the other PRRs, TLRs are capable of recognizing and responding to specific MAMPs, such as: LPS, flagellin, and dsRNA. Activation of TLRs results in specific changes in the modulation of events such as the regulation of proinflammatory cytokine production, cytokine mediated T cell maturation, and activation / proliferation of cellular immune responses (Purcell *et al.*, 2006; Rebl *et al.*, 2010; Foey and Picchietti, 2014). Several homologues to mammalian TLRs have been found in teleost fish, though their representation is not consistent across species. Additionally, the teleost TLRs are commonly functionally distinct from their mammalian counterpart, with many having unknown or unclear ligand specificity. The TLR genes identified in rainbow trout include: TLR1, TLR3, TLR5M, TLR5S, TLR7, TLR8a1/a2, TLR9, TLR20, and TLR22 (Palti *et al.*, 2006; Palti, 2011; Foey and Picchietti, 2014). Of these, TLR3 is known to be related to antiviral immunity and the TLR5 homologues are known to be specific for detection of flagellin. The ligand specificity of the remaining family members is either unknown or unproven (Rodriguez *et al.*, 2005; Palti, 2011; Foey and Picchietti, 2014). It is likely that many of the TLR homologs have ligand specificity in rainbow trout which correlates either with other closely related species or with that of their mammalian counterpart, but it has yet to be thoroughly investigated.

Nuclear factor *kappa*-light-chain-enhancer of activated B cells (NF-κB) is a transcription factor crucial to the regulation of the immune response. It is so important because it is a type of fast-acting transcription factor present in a suppressed form in cells, which does not require *de novo* protein synthesis in order to be activated or take effect. This property allows NF-κB to respond very rapidly to external stimuli. Known activators of NF-κB are variable but include tumour necrosis factor-*alpha* (TNF-α), interleukin-1 (IL-1), LPS, and flagellin. Pattern recognition receptors commonly make use of NF-κB as their effector to regulate changes in gene transcription governing the production of inflammatory cytokines, mediators of apoptosis, and

other important factors. As a result, the activation or suppression of NF-κB is a critical component of the signalling system involved in regulation of the immune system as a whole (Hayden *et al.*, 2006; Purcell *et al.*, 2006; Rebl *et al.*, 2010).

2.4.5 Inflammatory Response

Intestinal inflammation and the inflammatory response in general consist of a coordinated series of events, involving the individual mechanisms described above. The synchronised action of many systems results in a unified response to perceived threats, which is a vital component of the immune system and essential for survival. Inflammation can be characterized as being either acute or chronic and can be triggered by a number of external stimuli, such as physical trauma, pathogenic organisms, or irritants. For the purposes of this discussion, we will focus on the acute response since it is thought to be the significant process involved. The cardinal signs of an acute inflammatory response are something familiar to everyone and include: *Rubor* (redness), *Tumour* (swelling), *Calor* (heat), and *Dolor* (pain). These four cardinal signs were originally described almost 2000 years ago by early Greek and Roman scientists and are well known to the human medical and scientific establishment (Winn *et al.*, 2006). Unfortunately, in the case of our research, these signs are difficult, if not impossible, to directly observe in the gut of an organism particularly in the case of a sub-acute reaction. As such, we must infer the presence or absence of an inflammatory response indirectly, requiring a detailed understanding of the processes and mediators involved.

The diverse group of cells and other structures making up the immune system of the gut are collectively referred to as gut-associated lymphoid tissue (GALT). This system in teleost fish is less organized and functionally distinct from its mammalian counterpart, lacking common components such as Peyer's patches, M cells, IgA, and lymph nodes (Rombout *et al.*, 2011; Foey and Picchietti, 2014). The lack of these components means that these animals surely do not possess the type of mucosal immunity found in mammals. There is, however, a diverse population of immune cells present in the intestinal epithelium and lamina propria, including: macrophages, neutrophils, eosinophils, B cells, plasma cells, and intraepithelial lymphocytes (Rombout *et al.*, 2011; Foey and Picchietti, 2014). These cells are strategically placed and make use of plasma membrane PRRs to detect and respond to perceived threats through degranulation, cytokine production, chemokine secretion, *et cetera*. Intraepithelial lymphocytes (IELs) are a

special class of resident T cell found dispersed throughout the intestinal epithelium, residing between the enterocytes, in direct contact with the intestinal lumen. Unlike other types of T cells, IELs do not require priming and are therefore capable of responding immediately to the detection of antigens, playing an important role in immune surveillance and mediation mucosal immune responses (Rombout *et al.*, 2011; Foey and Picchietti, 2014). IEL-associated cytotoxic effects and cytokine production are key components of the innate immune system and inflammatory response in the gut due to the fact that a primary, immediate, luminal response to potential threats can be affected in addition to further signalling (Rombout *et al.*, 2011; Foey and Picchietti, 2014). Plasma cells and B cells present in the intestinal mucosa are responsible for the production of mucosal immunoglobulins. Unlike mammals, IgA is not found in teleosts; instead, IgM is the primary immunoglobulin, followed by IgD. A third novel Ig heavy chain has also been identified in teleosts, designated *tau* (τ) IgT in rainbow trout and *zeta* (ζ) IgZ in zebrafish. This novel Ig is expressed at the lowest level of the three known immunoglobulins and it is not entirely clear, at this point, what specific role it plays (Danilova *et al.*, 2005; Hansen *et al.*, 2005; Salinas *et al.*, 2011).

Once primary activation has occurred, regardless of the method, a similar cascade of events follows, propagating the inflammatory response. Plasma-derived and cell-derived mediators are both responsible for driving the propagation of inflammation. By the methods discussed in the preceding sections, a number of processes both stimulate and mediate each other. These processes have already been described in detail, so their individual contributions will only be briefly discussed here. Also, depending on the method by which the inflammatory response is triggered, the order of events and the relative contribution of each component can vary significantly. Since it is not practical to discuss all possible iterations of the process, a general overview will follow.

With respect to plasma-derived mediators, the complement system and coagulation system are likely participants in the mucosal inflammatory response. It would be intuitive to assume that the activation of these systems by luminal factors would require a physical breach of the epithelial barrier. Despite the lack of M cells, there is evidence that transepithelial antigen sampling does occur in teleosts, making a contribution of these systems a definite possibility (Fuglem, *et al.* 2010; Foey and Picchietti, 2014). Additional support, specifically for the role of complement, was found by Rawls *et al.* (2004), who showed transcriptional changes in

complement C3 in gnotobiotic zebrafish that were exposed to their first intestinal bacteria. In this event, the contributions of the complement system could include: C3a-stimulated histamine release from mast cells, C3b-mediated opsonisation of antigens, C5a-mediated chemoattraction of phagocytes and other immune cells, and MAC-mediated lysis of bacterial cells. The coagulation system, along with its function as a wound healing mechanism, can contribute to inflammation in a few ways as well. Thrombin, in addition to its role in clot formation, can bind to cells and prompt the production of both chemokines and nitric oxide (NO), which is toxic to bacteria (Szaba and Smiley, 2002; Touyz, 2007). Coagulation factor XII, when activated, contributes through the stimulation of both the kinin system and the fibrinolysis system which, in turn, contribute bradykinin and plasmin respectively. Bradykinin works to promote both vasodilation and vascular permeability, greatly increasing the transport of inflammatory mediators and cellular components to the site. Plasmin is a key mediator of both the complement and coagulation systems, working to cleave complement C3 and to activate coagulation factor XII, allowing for further propagation of the response. Plasmin is also responsible for keeping the coagulation system in check by facilitating the break-down of fibrin clots (Castellino and Ploplis, 2005; Golias *et al.*, 2007).

Cell-derived mediators are the second branch of the inflammatory response. A number of cell-types contribute to the initiation and propagation of inflammation. Mast cells and PMNs are key participants in the inflammatory response. Degranulation of these cell-types releases a large number of inflammatory mediators. These include: histamine, TNF-α, tryptase, interleukins, bradykinin, reactive oxygen species, enzymes, and chemokines. The combined function of these and other mediators either contribute to, or directly cause the signs of acute inflammation such as swelling, redness, heat, and pain. The recruitment of immune cells is accomplished through cytokine and chemokine production along with activation of key components in the complement and coagulation pathways. Additionally, local blood flow and vascular permeability are increased to speed delivery of blood-borne mediators and allow passage of phagocytes and platelets through tissues to the site. Stimulated degranulation of PMNs, including neutrophils and eosinophils, further contributes to the concentration of inflammatory, bactericidal, and antiviral mediators at the site further bolstering these effects (Kumar *et al.*, 2010; Rieger and Barreda, 2011; Foey and Picchietti, 2014).

Phagocytes also play an important role in the process. Some phagocytic cells are resident in the tissue and are part of initial activation, particularly macrophages. These cells play a dual role by actively working to seek out and destroy invaders while also producing chemokines and cytokines to further activate defences and attract reinforcements to the site. Cytokines such as TNF-α, IL-1, and IL-8 are contributed primarily by macrophages and exert many important effects. These can include stimulation of further cytokine production in other cells, upregulation of proinflammatory genes, chemotaxis of phagocytes and other immune cells, and activation of immune cells. Some of these cytokines are also responsible for systemic effects such as reduced appetite, fever, decreased blood pressure, and decreased heart rate. Additionally, the action of resident phagocytes serves to complement and enhance PRR activity in the intestinal epithelium by providing a supplementary and redundant primary alert, recruitment, and defence mechanism (Murray and Wynn, 2011).

Interferon production is also a contributor to inflammation, in addition to its roles in activation and regulation of cellular immunity. IFN-γ is produced primarily by natural killer-like (NK-like) cells and T cells. Increased IFN-γ results in activation or stimulation of NK-like cells, macrophages, and neutrophil activity. It also stimulates increased production of TNF-α and reactive oxygen species such as nitric oxide (NO). Type I interferon is also thought to contribute to inflammation, particularly chronic inflammation, possibly through the stimulation of cytokine and chemokine production (Ivashkiv, 2003; Robertsen, 2006; Giordano *et al.*, 2010).

The action of some mediators of inflammation also has the potentially negative side-effect of causing damage to host tissues. Toxins, reactive oxygen species, and other mediators released from granulocytes and phagocytes, targeted at the destruction of invaders, are equally capable of host tissue destruction. These actions are of particular concern when chronic inflammation develops and associated tissue damage can become significant and wide-spread. This fact necessitates the tight regulation of the inflammatory process through inhibitory and negative feedback mechanisms (Nathan and Ding, 2010). One such measure of control is the anti-inflammatory cytokine, interleukin 10 (IL-10). The factors governing the production and regulation of IL-10 are complex. It is produced and regulated differently in several cell types, including: B cells, T cells, and macrophages. The importance of IL-10 is rooted in its ability to regulate the level of inflammatory response and react to activation at several distinct levels. This

is achieved through modulation of effectors, antagonists, receptors, and immune cells themselves (Moore *et al.*, 2001).

IL-10 down-regulates the expression of several inflammatory effectors and mediators, including chemokines and proinflammatory cytokines, such as: IL1β, TNFα, and IFN-γ. Suppression of these substances not only directly contributes to a reduction in inflammation but also impairs the function of T cells, NK cells, and macrophages by blocking their signalling pathways and effectively slowing the progression of an inflammatory response. IL-10 is also capable of inhibiting antigen presentation through downregulation of MHC class II antigens on antigen-presenting cells. Additionally, upregulation of alternate suppressive mechanisms, such as interleukin-1 receptor antagonist (IL-1RA) or reduced expression of cytokine receptors like IL-1R, further impacts the system's capacity to respond to activation. Immune cell regulation results from the influence of IL-10 on the differentiation and/or growth of a wide range of immune cells, including: mast cells, PMNs, dendritic cells, NK cells, and many others (Moore *et al.*, 2001; Foey and Picchietti, 2014).

At the outset of this project, a single IL-10 gene was known to exist in rainbow trout. As of 2011, a second IL-10 paralogue was reported by Harun *et al.* (2011). The two paralogues are identified as tIL-10a and tIL-10b. The exact relationship of these two IL-10 genes with each other is not definitively known, nor is their relative importance to immune modulation in rainbow trout. It is likely that this pairing is another example of ohnologues retained from the most recent (Ss4R) whole-genome duplication event (Berthelot *et al.*, 2014). Sequence analysis by Harun *et al.* (2011) revealed that tIL-10a and tIL-10b share 92% identity in their coding regions, but share only 50% identity in their flanking 3′ and 5′ untranslated regions. This suggests two things, firstly that these paralogues likely share very similar effector functions and receptor specificity. Secondly, that the factors influencing post-transcriptional regulation of these two genes may differ greatly, potentially offering more specificity, flexibility, and greater control over total IL-10 activity (Harun *et al.*, 2011). It is also a possibility that one of these paralogues is in the process of being lost or inactivated as a result of the ongoing diploidization of the rainbow trout genome (Berthelot *et al.*, 2014).

2.5 Overview of Antinutritional Factors in Plant-based Feed Ingredients: Potential Impacts on Intestinal Health and Performance

Antinutritional factors (ANFs) can be defined as naturally occurring substances, found in feed ingredients, which have the potential to negatively impact growth, performance, and welfare when included in the diet (Francis *et al.*, 2001). There are a wide range of compounds present in plant-based protein sources which could potentially be ANFs. It is essential to consider that many of these substances are common to several ingredient sources, in varied amounts. In some cases, these compounds may be present in concentrations too low to be significant. Additionally, some ANFs are reduced, removed, or inactivated during processing of the raw crops into meal, production of protein concentrate ingredients, and production of extruded feed pellets. This review will focus on a selection of key antinutritional factors considered to be significant in plant-based feed ingredients for salmonid diets.

The main classes of antinutritional factors considered, due to their presence in the ingredients investigated here, include: phytates, glucosinolates, tannins, isoflavones, saponins, and proteinaceous allergens. Carbohydrate fractions of the experimental diets will also be examined due to their potential for negative impacts. These fractions include starch and non-starch polysaccharides (NSP). Each of these factors will be briefly described, detailing their functional properties and individual significance, in terms of their potential impact on the growth, performance, and welfare of salmonids.

Phytic acid, the common term for inositol hexakisphosphate (IP6), is found at varying levels in all of the raw plant-based feed ingredients being used for this study. The primary catabolite of IP6, inositol pentakisphosphate (IP5), is also found in appreciable levels. Phytic acid is produced by most plants as their primary storage form of phosphorus and is consequently found in the tissues and seeds. Though this compound is a significant source of phosphorus, it is not digestible by salmonids or other non-ruminant animals, since they lack any of the phosphatase enzymes necessary to break it down. Due to its physicochemical properties, phytic acid chelates divalent cations such as zinc (Zn^{2+}), iron(II) (Fe^{2+}), magnesium (Mg^{2+}), and calcium (Ca^{2+}) in the gut, preventing their absorption and resulting in reduced mineral availability and potential nutrient deficiencies (Duffus and Duffus, 1991; Francis *et al.*, 2001). A significant negative impact on protein digestibility is also possible, based two major mechanisms. The first is the formation of IP6-protein complexes which can result in significant

reduction protein solubility and restrict access of digestive enzymes (Cosgrove, 1966; Anderson, 1985; Morales *et al.*, 2011). The second mechanism is a reduction of both trypsinogen activation and the stability of trypsin, which combine to produce an overall reduction in trypsin activity (Singh and Krikorian, 1982; Inagawa *et al.*, 1987; Caldwell, 1992; Morales *et al.*, 2011)

It has also been shown that IP6 can have a significant effect on endogenous losses resulting from increased mucus production and secretion of endogenous amino acids. Research in pigs and broiler chickens suggests an indirect mode of action. Binding of IP6 with dietary proteins results in the formation of IP6-protien complexes. These complexes are resistant to both acidic solubilisation and proteolytic digestion, which stimulates increased secretion of gastric HCl and pepsinogen (Vaintrub & Bulmaga, 1991; Cowieson *et al.*, 2004; Bedford and Cowieson, 2009). This stimulated hypersecretory state results in increased gastric mucin secretion, in response to reduced pH, and an elevated concentration of pepsin (Kies, 2005; Bedford and Cowieson, 2009). Gastric emptying has a similar effect in the intestine, causing increased mucin and bicarbonate secretion to maintain an optimal environment (Cowieson *et al.*, 2004; Cowieson & Ravindran, 2007; Bedford and Cowieson, 2009).

Supplementation of salmonid diets with IP6 has been shown to have a detrimental effect on growth performance in both Chinook salmon (*Oncorhynchus tshawytscha*) (Richardson *et al.*, 1985) and rainbow trout (Spinelli *et al.*, 1983). Enzyme treatments are available to break-down the phytic acid found in feed ingredients, making it a good source of dietary phosphorus and reducing its potential for negative impacts. In pigs, the use of phytase has been shown to decrease gastric mucin secretion by as much as 25%, compared to untreated diets (Kies, 2005). Unfortunately, enzymatic treatments can be cost-prohibitive for producers to implement and are not currently optimized for use in aquafeeds (Morales *et al.*, 2011).

Glucosinolates are a class of goitrogenic glycoside compounds. They are produced as secondary metabolites, almost exclusively by plants of the order Brassicales. This order includes the papaya family (Caricaceae), the caper family (Capparaceae), and the mustard / cabbage family (Brassicaceae) of which canola is a member (Kjær, 1960; Ettlinger and Kjær, 1968; Sørensen, 1990; Rodman *et al.* 1996). In the plant, these compounds serve as a defence mechanism against insects, pathogens, and herbivores. The enzyme myrosinase, also called thioglucoside glucohydrolase, is produced in the plant along with glucosinolates, which together form the glucosinolate-myrosinase defence system. Glucosinolates are not toxic or harmful in

their intact state but the action of myrosinase triggers their defensive capabilities. The enzyme is compartmentalized within plant tissues in order to prevent damage to the plant. It is stored alongside glucosinolates such that the two compounds will interact in the event that tissue damage occurs. The resulting hydrolysis of glucosinolates by myrosinase cleaves a glucose moiety from the remainder of the compound. The residual portion of the glucosinolate compound is then converted into toxic secondary products, mainly thiocyanate ions, but also isothiocyanates, nitriles, goitrin, or oxazolidine-2-thiones under the right conditions.

The primary product of this reaction, thiocyanate, is responsible for the antinutritional properties commonly associated with glucosinolates. Thiocyanate is a goitrogen which functions by inhibiting iodine uptake by the thyroid gland and ultimately suppresses thyroid function through reduced production of the hormone thyroxine (T4) which is crucial for the regulation of both metabolism and growth. The other potential products of glucosinolate hydrolysis are also goitrogenic in nature and would have a comparable physiological impact if produced in sufficient quantities (van Etten and Tookey, 1979; Underhill, 1980; Larsen, 1981). With regards to toxicity, the breakdown products of glucosinolates, particularly isothiocyanates, are both highly reactive and potentially harmful. Strong exothermic reactions occur when these compounds are combined with a range of substances, including: amines, aldehydes, and phenols which may be present in the gut and can result in the production of toxic by-products (CAMEO, 2013). Regardless, these compounds are capable of causing severe irritation to the mucus membranes and intestinal epithelium on their own (Gaul, 1964; Fuller and McClintock, 1986; Brown, 2013). Oxazolidine-2-thiones are closely related to isothiocyanates and have the potential to produce similar reactions.

Nitriles are organic cyanides, a class of organic compounds which include a cyano (-CN) functional group. While significantly less toxic than isothiocyanates, nitriles are associated with growth and performance reductions as well as hepatic and renal damage in other species, such as: pigs, chickens, and rats (VanSteenhouse *et al.*, 1991; Francis *et al.*, 2001; Brown, 2013). These effects are thought to be mediated by oxidative stress and conjugation with hepatic glutathione (GSH). Significant effects on GSH levels in the liver and kidneys of rats have been observed, with rapid depletion in the liver followed by elevated hepatic and renal GSH levels. High concentrations of glucosinolate-derived nitriles can result in hepatic necrosis, bile duct hyperplasia, and necrosis of renal proximal tubules (VanSteenhouse *et al.*, 1991; Brown, 2013).

Tannins are large polyphenolic compounds found in many species of plants. These compounds are characteristically bitter or astringent and have the potential to significantly affect the palatability of diets. In terms of their antinutritional properties, the molecular structure of tannins includes a large number of hydroxyl (-OH) or carboxyl (-COOH) groups which allow these compounds to form strong bonds with proteins and other macromolecules (Cannas, 2013). Binding of proteins and other molecules, such as amino acids and alkaloids, by tannins results in their precipitation from solution and prevents further digestion or absorption by blocking the action of enzymes and transport mechanisms. Tannins also have the ability to chelate mineral ions, such as iron(II) (Fe^{2+}), preventing their absorption and potentially resulting in nutrient deficiencies (Liener, 1989; Francis *et al.*, 2001). The overall antinutritional action of tannins is to reduce the bioavailability of proteins and minerals in the diet, resulting in decreased nutrient digestibility and potentially impacting performance. Additionally, toxic effects can include damage to the intestinal epithelium and increased endogenous losses attributed to excretion of proteins and essential amino acids, likely related to increased mucin production (Bernays *et al.*, 1989; Cannas, 2013). It has also been proposed by Blytt *et al.* (1988) that tannins are relatively insensitive to interaction with membrane-bound proteins, such as digestive enzymes and absorptive mechanisms, suggesting that the primary impacts stem from other mechanisms.

Isoflavones are organic compounds found mainly in members of the Fabaceae family, which includes both soybeans (*Glycine max*) and field peas (*Pisum sativum*); however, only soybean meal contains a significant concentration. In the plant, these compounds serve as a defence mechanism, mainly against viral and fungal infections. When ingested, these compounds can have a much different effect. Classified as xenoestrogens or phytoestrogens, dietary isoflavones have the ability to act as agonists for both estrogen receptors and peroxisome proliferator-activated receptors (PPARs), among other activities (Medjakovic *et al.*, 2010; Penumetcha and Santanam, 2012). The two main isoflavones found in soybean meal are genistein and daidzein, both capable of activating all three PPAR types as well as estrogen receptors. The role of PPAR receptor ligands in inflammation and immunity is not fully understood. It has been demonstrated, mainly through *in vitro* and gene knockout studies, that PPARs play an integral role in the regulation of transcription factors, control of cytokine release, barrier permeability, and T-cell regulation. In general, an attenuation or measure of control over inflammation through activation of PPARs has been observed, suggesting that their activation is

not likely to have detrimental (pro-inflammatory) effects (Clark, 2002; Arnold and König, 2006; Mazzon *et al.*, 2009; Wang *et al.*, 2011; Di Paola, 2011). Isoflavones also have the potential to negatively affect both renal and hepatic estrogen metabolism as well as cholesterol uptake (Ng *et al.*, 2006). The overall impact and significance of these actions is not fully understood, but they have the potential to negatively affect both estrogen metabolism and growth performance while conversely having the potential to be beneficial for control of the inflammatory response.

Saponins are amphipathic glycoside compounds produced as secondary metabolites by many plant species including soybeans, peas, and canola. These compounds are classified by their physical property of soap-like foaming when agitated in an aqueous solution. Saponins are actually quite deadly to fish when added to water, due to their effect on respiratory cells of the gill epithelium. In fact, saponins are the active ingredient in some piscicidal compounds used specifically as poisons to kill fish (Milgate and Roberts, 1995; Francis *et al.*, 2001). Like some of the other compounds described, saponins are thought to be produced by plants as a defensive measure against microbial infection, fungal infection, and herbivores. Saponins have several relevant biological activities when consumed by animals. Due to their chemical structure and amphipathic nature, saponins have the ability to interact with both cholesterol and plasma membranes. Some saponins are capable of forming insoluble complexes with cholesterol, resulting in inhibition of cholesterol absorption from the gut. This property is exploited by the food production industry, where saponins can be used as an agent for the removal of cholesterol from food products (Micich *et al.*, 1992; Richardson *et al.*, 1994; Sundfeld *et al.*, 1994). The complexes that saponins form with cholesterol also give them the ability to permeabilize cell membranes, resulting in leakage and lysis of cells, including the cells of the intestinal epithelium and the blood. This action can result in damage to the intestinal epithelium and lysis of red blood cells, classifying saponins as hemolytic compounds. It is probable that this type of damage could have a significantly detrimental effect on intestinal barrier function and contribute to the initiation or propagation of an inflammatory response in the gut. A second property of saponins further affects nutrient absorption from the gut through binding and sequestration of bile salts, classifying them as a non-systemic bile acid sequestrant (Milgate and Roberts, 1995; Francis *et al.*, 2001; Francis *et al.*, 2002). The reduction in bile salt availability has the potential to significantly impact the capacity for micelle formation and consequently the absorption of lipids and lipid-soluble vitamins. Additionally, saponins have been observed to cause reductions in

both intestinal motility and protein digestibility, further impacting their possible negative effects on health, welfare, and performance of animals (Francis *et al.*, 2002). Any significant reduction in intestinal motility also has the potential to impact intestinal microbiota by allowing for greater fermentative activity and increasing the time and opportunities for colonization. These combined effects have the potential to significantly impact the structure and diversity of the microbial population in the gut.

Glycinin (11S globulin) and β-conglycinin (7S globulin) are the major storage proteins found in soybeans. These proteins or peptides derived from them, are resistant to digestion and possess physicochemical properties associated with an allergic hypersensitivity reaction (Pedersen, 1989; Lalles and Peltre, 1996; Lalles *et al.*, 1999). Rumsey *et al.* (1994) showed a clear link between these antigenic proteins and the detrimental effects commonly associated with soybean meal inclusion in salmonid diets. They observed significant negative associations with growth rate, feed conversion, and protein utilization in rainbow trout. They also observed the significant inflammatory response, increased phagocytic activity, and changes in the mucosal morphology of the gut which are now synonymous with inclusion of high levels of soybean meal in salmonid diets (Rumsey *et al.*, 1994). This effect, termed soybean meal-induced enteritis by Baeverfjord and Krogdahl (1996), has a range of symptoms, including: shortening of intestinal villi and microvilli, marked decrease or loss of normal supranuclear vacuolization of enterocytes, widening of the lamina propria, and increased infiltration of inflammatory cells into the submucosa. Large reductions in the levels of digestive enzymes have also been observed, both of the brush border and those secreted by intestinal epithelial cells. The overall impact of this condition is a significant reduction in the digestive and absorptive capacity of the intestinal epithelium, presenting with decreased nutrient digestibility, increased feed conversion ratio, and reduced growth (Burrells *et al.*, 1999; Bakke-McKellep *et al.*, 2000; Buttle *et al.*, 2001; Krogdahl *et al.*, 2003; Sanden *et al.*, 2005).

Similar potentially antigenic proteins also exist in peas and canola. Legumin (11S globulin), vicilin (7S globulin), and convicilin (7S globulin) are the major storage proteins found in peas. Cruciferin (11S globulin) and napin (2S albumin) are the major storage proteins found in canola. These proteins are similar in structure to their counterparts, found in soybeans, and are also potential allergens. Several studies have investigated the potential allergenic properties of these proteins, mainly in humans. There is currently no data on the development of allergic

hypersensitivity reactions to these proteins in teleost fish, but it nevertheless remains a possibility. An additional point to consider is the potential for cross-reactivity of antibodies, which has been demonstrated in humans. Antibodies generated in response to an 11S or 7S globulin from one species of plant have the potential for activity against closely related proteins from other plant species (Monsalve *et al.*, 1997; Mohamed Salleh *et al.*, 2002; Sanchez-Monge *et al.*, 2004; Abeysekara, 2012; Bar-El Dadon *et al.*, 2013).

Dietary carbohydrates also play a potential role as antinutritional factors, particularly in species that are not well-adapted to their consumption. Carbohydrates found in the diet fall into two broad classifications, starch and non-starch polysaccharides (NSPs). Starch is a common polysaccharide found in all green plants, used for storage of glucose produced by photosynthesis. With respect to the plant-based protein sources of interest to the aquaculture industry, it is found with particular abundance in field peas. As discussed earlier, carnivorous teleosts are not well adapted to consuming high levels of starch in their diet. Comparatively low levels of essential digestive enzymes and reduced capacity for adaptation to changing levels of starch in the diet make it difficult for salmonids to adjust to high starch diets. Such diets are known to result in poor utilization of dietary energy, decreased macronutrient digestibility, and reduced growth of rainbow trout (Hilton *et al.*, 1983; Thiessen *et al.*, 2003).

Non-starch polysaccharides (NSPs) can be classified as either soluble or insoluble. Speaking generally, elevated levels of soluble high molecular weight NSPs, such as glucans and arabinoxylans, can have several affects, including: increased viscosity of digesta due to their water-holding and gel-forming properties, delayed gastric emptying, decreased passage rate, chelation of cholesterol and divalent cationic minerals (Fe^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}), and an overall decrease in nutrient digestibility (Almirall *et al.*, 1995; Choct *et al.*, 1996; Amirkolaie *et al.*, 2005). Many of these effects can be tied to a significant increase in the viscosity of digesta which slows passage rates, limits convection of digesta thereby reducing exposure to the intestinal epithelium, and decreases the overall efficiency of digestive and absorptive processes. It has been shown in other monogastric species that addition of soluble NSPs to the diet, in conjunction with increased digesta viscosity, results in increased fermentative activity in the proximal intestine, which significantly contributes to the antinutritional properties of soluble NSPs (Choct *et al.*, 1996). Insoluble NSPs, such as cellulose, remain suspended in digesta and do not normally have a significant effect on viscosity (Smits and Annison, 1996; Amirkolaie *et al.*, 2005).

Additionally, high levels of insoluble fibre have a negative effect on the stability of feed pellets in water, which is of significant concern for aquafeeds (FAO, 1980; Lim *et al.*, 1997). The NSP fractions of soybean meal, pea meal, and canola meal consist mainly of pectic polysaccharides. The primary contributors are rhamnogalacturonans with varied side-chains of neutral sugars, including: arabinose, xylose, and galactose (Choct and Kocher, 2000; Meng and Slominski, 2005). It is also possible for indigestible dietary fractions, such as these, to have a significant impact on the diversity and composition of the intestinal microbial population by acting as a selective fermentation substrate and favouring the proliferation of those species that are best suited to utilize it. Studies in pigs, chickens, and humans have all demonstrated that NSPs can significantly affect the composition of the intestinal microbial population, which may not be a desirable outcome in all cases (Högberg *et al.*, 2004; Metzler-Zebeli *et al.*, 2010; Saki *et al.*, 2010; Bindelle *et al.*, 2011; Walker *et al.*, 2011).

A number of methods are available for analysis of dietary fibre. These methods vary in their ability to quantify specific fibres or their functional characteristics in feed ingredients. As a result, a major issue regarding estimation of the antinutritional effects of fibre relates to the specific analytical method employed. Detergent fibre analysis is an improvement over the older crude fibre method, which came into widespread use in the 1970's (Fahey *et al.*, 2011). Acid detergent fibre (ADF) and neutral detergent fibre (NDF) are the two assays performed during this type of analysis. Though still widely used as the standard fibre analysis for many scientists, these values do not provide a precise picture of total dietary fibre, since the soluble fractions are not accounted for and overlapping detection of insoluble fibres exists between the methods (Fahey *et al.*, 2011). Nevertheless, a useful representation of fibre composition can still be obtained and these assays continue to be widely used. More recently, methods of total dietary fibre analysis have come to the forefront and are capable of producing a much more accurate picture of the fibre content of diets or ingredients. These methods improve upon crude fibre and detergent fibre analysis through quantification of the soluble fraction and provide more detailed information about fibre content than was previously attainable. Modified detergent fibre analysis is well suited to the rapid study of the insoluble fraction of dietary fibre but total dietary fibre methods, such as the enzymatic-gravimetric and enzymatic-chemical methods are much better at providing a complete picture of total dietary fibre (Fahey *et al.*, 2011).

3.0 OBJECTIVES AND HYPOTHESIS

This study investigated the impact of several alternative protein sources on the intestinal physiology of rainbow trout, in correlation with growth performance. Soy, pea, and canola-based protein sources were investigated at varied levels of inclusion and at two distinct levels of processing, a meal and a protein concentrate. The meal was minimally refined and was expected to retain many of the putative ANFs present in the raw seed. Conversely, the processes of alcohol extraction (soy), aqueous extraction (canola), and air fractionation (pea) utilized to produce purified protein concentrates were expected to reduce or eliminate these ANFs, in addition to greatly increasing the relative protein content of these ingredients (Collins *et al*., 2013). It was anticipated that comparisons of performance and gastrointestinal responses among these diverse protein sources would permit conclusions about factors limiting inclusion of such ingredients in the diets of farmed fish. This information would be beneficial in providing strategies for the increased commercial utilization of these ingredients in the future.

The practical goal of this research was to provide information toward the determination of an optimal plant-based ingredient selection, processing requirement, and inclusion rate in aquaculture diets for rainbow trout. It is hoped that this will ultimately allow for the substitution of a significant quantity of fish meal from existing diet formulations, alleviating at least a portion of the current constraints on long-term expansion of the aquaculture industry. It is important that this goal is met while also maintaining desirable performance parameters and without negatively impacting the health or welfare of the fish.

It is our hypothesis that the correlation of physiological changes in the gastrointestinal tract of rainbow trout to the degree of processing and inclusion level of different plant-based ingredients in the diet will help to identify plant components mediating adverse effects on the intestinal epithelium. Based on this hypothesis, our long-term objective is to contribute to research which will determine the specific factors and physiological pathways limiting inclusion of plant-based ingredients in salmonid diets and aid in the provision of strategies to increase their utilization as a source of high quality protein for commercial aquafeeds.

Our specific technical objectives for the current study include the following: examination of physiological responses to dietary inclusion of physicochemically different plant-based protein sources at two levels of processing and at different levels of inclusion; determination of

correlations between the dietary inclusion of specific antinutritional factors, abundance of mRNA transcripts for selected genes in the intestine, and growth performance; the development of recommendations regarding the suitability of the experimental ingredients for commercial aquaculture diets.

4.0 MATERIALS AND METHODS

4.1 Animal Husbandry

Feeding trials were conducted at the Prairie Aquaculture Research Centre (University of Saskatchewan, Saskatoon, SK, Canada), a biologically filtered, semi-closed recirculating aquaculture system. The fish used for these experiments were triploid female rainbow trout, acquired from Wild West Steelhead (Lucky Lake, SK, Canada). Water temperature was maintained at 15 ± 1 °C and the photoperiod was 14 h light : 10 h dark. Environmental and water quality indicators were closely monitored over the course of the experiments. A commercial fish meal based diet was fed for two weeks prior to the initial experiment in order to acclimatize the fish to the environment. This diet was also fed for a minimum of one week between trials, since the same group of fish was used for all six growth trials. The guidelines set by the Canadian Council on Animal Care were followed in the maintenance of all fish for the duration of this trial (CCAC, 1993; CCAC, 2005).

4.2 Experimental Design

A total of six experimental ingredients were tested, including: pea meal (PM; yellow field pea, CDC Mozart, Crop Development Centre, Saskatoon, SK, Canada), air fractionated pea protein concentrate (PPC; yellow field pea, prestige protein, Parrheim Foods, Saskatoon, SK, Canada), soybean meal (SBM; Federated Cooperatives Limited, Saskatoon, SK, Canada), aqueous ethanol extracted soy protein concentrate (SPC; Soycomil K; ADM Specialty Ingredients (Europe) BV, Koog aan de Zaan, The Netherlands), canola meal (CM; canola meal-35; Federated Co-Operative Ltd., Saskatoon, SK, Canada), and a dephytinized canola protein concentrate produced by aqueous extraction (CPC; Can Pro IP; CanPro Ingredients Ltd., Arborfield, SK, Canada). The feeding trials were conducted over the course of one year. The order of plant protein testing was randomized and each plant protein meal and its respective protein concentrate were tested in succession and relative to a zero inclusion reference diet (Table 4.1).

Table 4.1 Fish distribution per tank, chronological progression, and average starting weight (g) for the six feeding trials.

Ingredient	Fish (n=)	Start (d)	End (d)	Average start weight ± SD
PM	22	0	56	235.2 ± 17.1
PPC	22	1	57	237.7 ± 21.7
SBM	17	130	186	553.0 ± 44.6
SPC	17	166	222	610.8 ± 22.7
CM	16	192	248	639.8 ± 77.5
CPC	17	305	361	824.6 ± 82.8

Each ingredient was tested through an independent growth trial, utilizing graded levels of inclusion from 0 (control diet) to 300 g kg^{-1}. The fish in each trial were fed three energetically and nutritionally identical diets with increasing levels of the test ingredient (0, 150, and 300 g kg^{-1}) to determine their effect, if any, on performance, health, and welfare in relation to inclusion level. The control diet was formulated based on the purified reference diet reported by Cho *et al.* (1985). This diet was slightly modified by the addition of Celite® 545 high-purity flux-calcined diatomaceous earth (Celite Co., World Minerals Co., Lompoc, CA, USA) as a non-absorbable indicator for indirect digestibility analysis. The control diet (0 g kg^{-1}) and the maximum inclusion (300 g kg^{-1}) diet were formulated independently and subsequently combined in relative proportions to produce the 150 g kg^{-1} diet. The diets were formulated to be isoenergetic and isonitrogenous, on a digestible nutrient basis, containing 17.6 MJ kg^{-1} digestible energy and 386.2 g kg^{-1} digestible crude protein (Collins *et al*. 2012). Diets were balanced for essential amino acids according to Mambrini and Guillaume (1999) and either met or exceeded rainbow trout nutrient requirements (NRC, 1993).

Diets were mixed on a Hobart mixer (Hobart Corporation; Model L-800; Troy, OH, USA) and by hand, as needed, then cold extruded through a 5-mm die on a Hobart mixer (Hobart Corporation; Model 4822; Troy, OH, USA), dried in a forced air oven for 12 h at 55ºC, then chopped and screened to form pellets of uniform size.

A commercial aquaculture diet was fed for a period of two weeks preceding the first trial in order to adapt all of the fish to their environment. Due to the fact that the same group of fish

was maintained across all six trials, the commercial diet was also fed for at least one week between experiments as a measure of normalization. For each 56-day trial, nine 360 L tanks were used, with three replicates per treatment. The diets were randomly assigned to the tanks and hand-fed twice daily to apparent satiation. For every trial, each tank of fish was weighed on days 0 and 56 and total feed intake was measured. Following the feeding period, on day 57, three fish were taken from each tank and humanely euthanized by a sharp blow to the cranium prior to dissection. Sub-sampling was conducted with the aim of collecting average-sized animals from each tank, avoiding extremes in body size.

4.3 Sample Collection

Gross anatomical data for each animal including body weight, liver weight, and overall gut length, from the termination of the pyloric stomach to the vent, were recorded. The isolated intestine of the animal was subdivided into two main sections: mid-gut = section from the last pyloric cecum to the beginning of the distal gut (visual abrupt increase in diameter or 50% of the distance from last pyloric caeca to the vent) and distal gut = section from the end of the mid-gut to the vent.

Histological samples, approximately 4 mm in length, were collected from an undisturbed region of the distal gut. Samples were cut directly into tissue cassettes, with minimal handling, and fixed in 10% neutral buffered formalin. Digesta samples were collected aseptically from each intestinal section and stored on ice before transfer into cold storage at -80°C. Following collection of digesta, the remaining tissues were collected for analysis of mRNA transcript abundance. Samples were individually placed into Whirl-Pak® bags and snap-frozen in liquid nitrogen. To maintain RNA integrity, snap-frozen samples were stored on dry ice before transfer to cold storage at -80°C.

4.4 Analysis of mRNA Transcript Abundance by qPCR

Snap-frozen intestinal samples were manually ground to a fine powder with a mortar and pestle, under liquid nitrogen freezing, prior to RNA extraction. AbSolve™ Glassware Cleaner (Perkin Elmer, Shelton, CT, USA) was used to remove potential RNase contamination from tools prior to grinding. Following grinding, tissue samples were stored in sterile 15 ml tubes at -80°C.

Total RNA was extracted from 30 - 50 mg of ground intestinal tissue using TRI Reagent® Solution (Applied Biosystems / Ambion, Inc., Austin, TX, USA). Extracted RNA was subjected to an RNase-free DNase digestion (TURBO DNA-*free*™, Applied Biosystems / Ambion, Inc., Austin, TX, USA) and stored at -80°C. The RNA content of the extracted samples was assessed using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). Randomly selected samples were also tested using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) in order to confirm RNA purity / integrity and assess quality variation among samples. One μg of total RNA was then used to generate first strand cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA, USA).

Oncorhynchus mykiss-specific qPCR primers were designed using Beacon Designer™ 4.0 (PREMIER Biosoft International, Palo Alto, CA, USA) from genomic DNA or mRNA sequences obtained from the National Center for Biotechnology Information (NCBI) nucleotide database. If suitable genomic sequence was available, primers were targeted to intron-spanning regions in order to prevent amplification of genomic DNA. All utilized primer sets were designed *de novo*, with the exception of those for elongation factor 1α, which were obtained from Kirchner *et al.* (2008) and validated *in silico* using the complete mRNA coding sequence along with the primer design software. The gene targets chosen for analysis were the housekeeping genes *beta*-actin (GenBank: AF157514.1), 18S rRNA (GenBank: AF308735.1), and elongation factor 1α (GenBank: AF498320.1), in addition to the targets immunoglobulin M (GenBank: X83372.1), interleukin-1 *beta* (GenBank: AJ004821.1), interleukin 8 (GenBank: AJ310565.1), interleukin 10 (GenBank: AB118099.1), and proliferating cell nuclear antigen (GenBank: CA359986.1). The 780 bp *Oncorhynchus mykiss* mRNA sequence CA359986.1 shared 100% sequence identity with *Salmo salar* proliferating cell nuclear antigen putative mRNA (GenBank: BT056695.1, *Salmo salar* clone ssal-evd-518-241) and was presumed to be *Oncorhynchus mykiss* proliferating cell nuclear antigen. *Beta*-actin (β-act), 18S rRNA, and elongation factor 1α (EF-1α) were all screened as candidates for use as an internal control for normalization of qPCR data.

Primers were verified for specificity using the blastn function of the Basic Local Alignment Search Tool (BLAST) to compare the primer sequences against the NCBI nucleotide database. Further, an annealing temperature gradient PCR was conducted (iCycler™ thermal

cycler, Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a reaction mixture containing 5 µl of 10× PCR buffer, 1.5 µl of 50 mM $MgCl_2$, 1 µl of each deoxyribonucleotide triphosphate (dNTP) at a concentration of 10 mM, 1 µl of each primer (25 µM), 0.2 µl of *Taq* polymerase, template cDNA isolated from intestinal samples, and nuclease-free 0.2 µm filtered water to a final volume of 50 µl. Cycling parameters were 95 °C for 5 min, followed by 30 cycles of 1.5 min consisting of 30 s at 95 °C, 30 s at 52 - 60 °C, and 30 s at 72 °C. Each sample was amplified in quadruplicate at four separate annealing temperatures (Ta), straddling the optimum Ta predicted by Beacon Designer™. The resulting PCR products were visualized by agarose gel electrophoresis in a 1.5% agarose gel containing 0.5 µg ml^{-1} ethidium bromide. Amplicon size was verified visually against a 1 Kb Plus DNA Ladder (Invitrogen Co., Carlsbad, CA, USA) and the brightest of the four bands was selected as the optimal annealing temperature. Bands were excised and subsequently extracted from the agarose gel using a QIAEX® II Gel Extraction Kit (QIAGEN, Inc., Germantown, MD, USA).

The gel-extracted PCR product was ligated into the pGEM® - T Easy Vector (Promega Co., Madison, WI, USA) and the resulting construct was used to transform JM109 competent *E. coli* cells. The transformed cells were cultured on Luria-Bertani (LB) media containing 100 µg ml^{-1} of ampicillin and top-dressed with 50 µl of isopropyl β-D-1-thiogalactopyranoside (IPTG, 200 mM), and 50 µl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 20 mg ml^{-1}). After an overnight incubation at 37 °C a single, isolated, successfully transformed (white) colony from each gene target was picked into 5 ml of LB broth containing 100 µg ml^{-1} of ampicillin using a sterile toothpick. The tubes were incubated overnight at 37 °C while shaking at approximately 150 rpm. Following incubation, the broth cultures were centrifuged at 2000 × *g* for 10 min to form a pellet. The cells were then processed with a QIAprep® Spin Miniprep Kit (QIAGEN, Inc., Germantown, MD, USA) to isolate purified plasmid DNA. Sequencing primers (pUC/M13) compatible with this cloning vector were utilized to sequence the purified plasmid DNA, in both the forward and reverse directions, on an ABI 3730 capillary sequencer (Applied Biosystems, Inc., Foster City, CA, USA). Sequence information was verified by alignment to the predicted amplicon sequences provided by Beacon Designer™.

For the transcript abundance assays, RT-qPCR was completed using the CFX96™ real-time PCR detection system with a C1000™ thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA). The reaction mixture contained iQ SYBR® Green Supermix (Bio-Rad), 500 nM of both the

forward and reverse primers, template cDNA reverse-transcribed from total RNA isolated from intestinal samples, and nuclease-free 0.2 µm filtered water to a final volume of 12.5 µl. Cycling parameters were: 95 °C for 3 min, followed by 40 cycles of 60 s consisting of 30 s at 95 °C and 30 s at the optimum annealing temperature for each primer set as shown in Table 4.2, followed by a melt curve stepping from 65 °C to 95 °C in 0.5 °C increments.

Table 4.2 *Oncorhynchus mykiss* specific primers used for qPCR analysis.

Target	Sequence (5′ - 3′)	Annealing Temp (°C)	Amplicon (bp)	Reference
βact	GCGACCTCACAGACTACC TACCGCAAGACTCCATACC	53	273	This Study
18S rRNA	TGTCTGCCCTATCAACTTTC CTTCCTTGGATGTGGTAGC	55	120	This Study
EF-1α	TCCTCTTGGTCGTTTCGCTG ACCCGAGGGACATCCTGTG	59	159	Kirchner *et al.* (2008)
PCNA	TGTGACCGCAACCTCGCAATGG CACGGCAGATACGGGCAAACTCC	57	265	This Study
IgM	CACTTCATCAGATGGTCCAGTCC ACAGTCCCATTGCTCCAGTCC	57	243	This Study
IL-1β	TGCTGTGGAAGAACATATAGTG ACGAAGACAGGTTCAAATGC	55	212	This Study
IL-8	CTGACCATTACTGAGGGGATGAG AATCTCCTGACCGCTCTTGC	60	180	This Study
IL-10	GAGACTACTACGAGGCTAATGAC TGTTGTTCTGTGTTCTGTTGTTC	60	165	This Study

For determination of transcript copy number, standard curves ranging from 10^1 - 10^7 copies of each target gene were made using the purified plasmid DNA. Plasmid DNA was quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and copy number was determined based on the size of each vector with its respective insert (bp) and the concentration of DNA. Transcript copy numbers are reported per 50ng of total RNA.

4.5 Protein and Enzyme Assays

Caspase-3 activity was determined using the EnzChek® Caspase-3 Assay Kit #1 *Z-DEVD-AMC Substrate* (Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's instructions.

Briefly, ground intestinal tissue was homogenized at a concentration of 5.0 mg per 200 µl in cell lysis buffer and aliquoted into two separate tubes. One of these aliquots was used for the caspase-3 assay and the other was used for a Bradford protein assay to quantify the total protein content of the solution. Caspase activity was assessed as fluorescence emission at 441 nm following excitation at 342 nm, after cleavage of the 7-amino-4-methylcoumarin (AMC)-derived substrate (Z-DEVD-AMC) in duplicate reactions. The assays were performed using 50 µl of tissue homogenate and quantified with a Fluoroskan Ascent® FL microplate fluorometer (Thermo-Labsystems, Franklin, MA, USA). Active caspase (µmol g^{-1} of protein) was determined by interpolation of fluorescence emission for homogenates against a dilution series of purified AMC reference standard and normalized to total protein content, assayed from the second aliquot of tissue homogenate (Bradford Protein Assay, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

4.6 Histological Examination of Distal Gut Tissues

Following sample collection, as described above, samples were allowed to fix for 24 - 48 hours. After fixation was complete, samples were delivered to Prairie Diagnostic Services (PDS), a full-service diagnostics laboratory located in the Western College of Veterinary Medicine on the University of Saskatchewan campus. Samples were processed, sectioned, and stained with hematoxylin and eosin by PDS according to their standard histological procedures. The mounted and stained distal gut sections were then examined using an Axiostar *plus* transmitted light microscope (Carl Zeiss Canada, Ltd., Toronto, ON) and measurements were taken using the accompanying AxioVision 3.1 digital image processing software (Carl Zeiss Canada).

4.7 Statistical Analysis

Transcript abundance data was processed using the 'Explore' descriptive statistics function of IBM© SPSS© Statistics 19.0.0. This procedure quantifies the central tendency and dispersion of a data set, which were used to identify outliers for exclusion, prior to further investigation. Outliers were defined, by SPSS©, as values deviating from the median by greater than 1.5× the interquartile range (SPSS Inc., Chicago, IL, USA). Statistical analysis of processed transcript abundance data was performed using the univariate general linear model (GLM) procedure of SPSS©. Independent experiments for each dietary ingredient (Table 4.1) were

analyzed separately with inclusion level (0, 150, and 300 g kg^{-1} diets) as the source of variation. In the case of significant ($P < 0.05$) variation in inclusion level, differences in means were determined by post hoc analysis using the Tukey's HSD test.

Pearson correlation coefficients were also calculated between selected variables, combining data from all independent experiments, using IBM© SPSS© Statistics 19.0.0 (SPSS Inc., Chicago, IL, USA). Data from additional variables including specific growth rate (SGR), average daily feed intake (ADFI), feed conversion ratio (FCR), protein efficiency ratio (PER), dietary ingredient inclusion, and dietary ANF content recorded from these experiments and previously reported by Collins *et al.* (2012; 2013) were also included. Significance of Pearson correlation results was determined when $P < 0.05$, non-significant trends were determined when $0.05 < P < 0.10$.

5.0 RESULTS

5.1 Transcript Abundance of Selected Internal Control Genes

Three internal control genes were evaluated for use in this study including β-actin, 18S rRNA, and EF-1α. Prior to conducting the canola-product trials, β-actin and 18S rRNA were selected for evaluation but the univariate GLM analysis of qPCR results from the pea-product and soy-product trials revealed statistically significant effects ($P < 0.05$) of inclusion level on their abundance for the PM, PPC, and SBM diets (Table 5.1, Table 5.2). Based on these results, it was determined that β-actin and 18S rRNA were not suitable for use as internal controls in this experiment and a new internal control gene, EF-1α, was selected for evaluation. Elongation factor 1-*alpha* was tested against all six experimental treatments and significant effects ($P < 0.05$) of inclusion level were found for the PM, SBM, and CPC diets (Table 5.3).

Based on these findings, an internal control gene could not be validated for use in this study. As a result, transcript abundance values are reported as transcript copy number per 50 ng of total RNA rather than transcript copy number per copy of an internal control gene.

Table 5.1 Mean (SD) abundance ($\times 10^7$ copies per 50 ng total RNA) of β-actin mRNA in distal intestine of rainbow trout fed diets formulated with increasing levels of pea or soybean protein at two levels of processing.

Ingredient [1]	Inclusion Level					
	0 g kg^{-1}		150 g kg^{-1}		300 g kg^{-1}	
	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI
PM	3.96 (0.93) [b]	(3.35, 4.57)	1.82 (1.11) [a]	(1.09, 2.55)	3.62 (1.08) [b]	(2.91, 4.33)
PPC	4.48 (0.45) [b]	(4.18, 4.78)	1.54 (1.41) [a]	(0.62, 2.46)	3.44 (0.66) [b]	(3.01, 3.87)
SBM	3.97 (1.47) [b]	(3.01, 4.93)	1.69 (1.02) [a]	(1.02, 2.36)	3.73 (1.20) [b]	(2.95, 4.51)
SPC	2.98 (2.82) [a]	(1.14, 4.82)	1.92 (1.17) [a]	(1.16, 2.68)	2.53 (1.44) [a]	(1.59, 3.47)

[1] PM, pea meal; PPC, pea protein concentrate; SBM, soybean meal; SPC, soy protein concentrate

[ab] Means in the same row with different superscripts are significantly different ($P < 0.05$)

Table 5.2 Mean (SD) abundance ($\times 10^3$ copies per 50 ng total RNA) of 18S rRNA in distal intestine of rainbow trout fed diets containing plant based ingredients at two levels of processing.

Ingredient [1]	Inclusion Level					
	0 g kg^{-1}		150 g kg^{-1}		300 g kg^{-1}	
	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI
PM	1.00 (0.12) [a]	(0.92, 1.08)	1.37 (0.06) [a]	(1.33, 1.41)	1.13 (0.15) [a]	(1.03, 1.23)
PPC	1.17 (0.27) [a]	(0.99, 1.35)	1.25 (0.03) [a]	(1.23, 1.27)	1.06 (0.21) [a]	(0.92, 1.20)
SBM	1.79 (0.21) [b]	(1.65, 1.93)	1.18 (0.30) [a]	(0.98, 1.38)	1.56 (0.15) [b]	(1.46, 1.66)
SPC	1.45 (0.06) [a]	(1.41, 1.49)	1.44 (0.06) [a]	(1.40, 1.48)	1.32 (0.03) [a]	(1.30, 1.34)

[ab] Means in the same row with different superscripts are significantly different ($P < 0.05$)

Table 5.3 Mean (SD) ($\times 10^2$ copies per 50 ng total RNA) abundance of EF-1α mRNA in distal intestine of rainbow trout fed diets containing plant based ingredients at two levels of processing.

Ingredient [1]	Inclusion Level					
	0 g kg^{-1}		150 g kg^{-1}		300 g kg^{-1}	
	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI
PM	9.12 (2.94) [ab]	(7.20, 11.04)	12.61 (5.09) [b]	(9.28, 15.94)	6.43 (0.91) [a]	(5.84, 7.02)
PPC	7.67 (2.70) [a]	(5.91, 9.43)	8.92 (5.94) [a]	(5.04, 12.80)	6.39 (1.86) [a]	(5.17, 7.61)
SBM	19.79 (3.45) [b]	(17.54, 22.04)	9.51 (3.75) [a]	(7.06, 11.96)	20.07 (3.42) [b]	(17.83, 22.31)
SPC	13.22 (9.36) [a]	(7.10, 19.34)	14.05 (6.57) [a]	(9.76, 18.34)	13.40 (5.97) [a]	(9.50, 17.30)
CM	3.48 (1.81) [a]	(2.30, 4.66)	5.28 (5.46) [a]	(1.71, 8.85)	6.12 (3.90) [a]	(3.57, 8.67)
CPC	12.95 (6.63) [b]	(8.62, 17.28)	6.49 (1.85) [a]	(5.28, 7.70)	10.22 (1.51) [ab]	(9.23, 11.21)

[1] CM, canola meal; CPC, canola protein concentrate

[ab] Means in the same row with different superscripts are significantly different ($P < 0.05$)

5.2 Transcript Abundance of Inflammatory and Immune Marker Genes

Transcript abundance of PCNA, a tissue marker used to indicate cell proliferation, in the distal gut is shown in Table 5.4. For the SBM treatment, a significant decrease in PCNA abundance was observed for the 150 g kg^{-1} diet, compared with the control and 300 g kg^{-1} diets. No other significant treatment effects were seen on the abundance of PCNA.

Table 5.4 Mean (SD) abundance ($\times 10^4$ copies per 50 ng total RNA) of PCNA mRNA in distal intestine of rainbow trout fed diets containing plant based ingredients at two levels of processing.

Ingredient [1]	Inclusion Level					
	0 g kg^{-1}		150 g kg^{-1}		300 g kg^{-1}	
	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI
PM	4.60 (2.70) [a]	(2.84, 6.36)	6.37 (3.66) [a]	(3.98, 8.76)	5.01 (1.59) [a]	(3.97, 6.05)
PPC	4.86 (2.22) [a]	(3.41, 6.31)	4.00 (2.79) [a]	(2.18, 5.82)	4.27 (1.33) [a]	(3.40, 5.14)
SBM	11.87 (2.52) [b]	(10.22, 13.52)	6.67 (4.38) [a]	(3.81, 9.53)	12.42 (4.29) [b]	(9.62, 15.22)
SPC	9.23 (7.23) [a]	(4.51, 13.95)	9.02 (6.42) [a]	(4.83, 13.21)	6.46 (3.42) [a]	(4.23, 8.69)
CM	0.79 (0.56) [a]	(0.43, 1.15)	1.58 (1.95) [a]	(0.30, 2.86)	3.03 (3.05) [a]	(1.03, 5.03)
CPC	3.90 (1.92) [a]	(2.65, 5.15)	4.64 (3.18) [a]	(2.56, 6.72)	3.43 (0.81) [a]	(2.90, 3.96)

[ab] Means in the same row with different superscripts are significantly different ($P < 0.05$)

IgM abundance was measured as an indicator of B cell infiltration and antibody secretion in distal gut lamina propria (Table 5.5). For the SBM treatment, a significant decrease in IgM abundance was observed for the 150 g kg^{-1} diet, compared with the 300 g kg^{-1} diet, but neither was significantly different from the control. No other significant treatment effects were seen for abundance of IgM.

Table 5.5 Mean (SD) abundance ($\times 10^4$ copies per 50 ng total RNA) of IgM mRNA in distal intestine of rainbow trout fed diets containing plant based ingredients at two levels of processing.

Ingredient [1]	Inclusion Level					
	0 g kg^{-1}		150 g kg^{-1}		300 g kg^{-1}	
	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI
PM	4.57 (4.17) [a]	(1.85, 7.29)	2.10 (1.50) [a]	(1.12, 3.08)	2.51 (1.70) [a]	(1.40, 3.62)
PPC	3.73 (2.49) [a]	(2.10, 5.36)	3.37 (3.09) [a]	(1.35, 5.39)	2.07 (1.19) [a]	(1.29, 2.85)
SBM	19.89 (11.61) [ab]	(12.30, 27.48)	6.78 (2.72) [a]	(5.01, 8.55)	33.44 (31.05) [b]	(13.15, 53.73)
SPC	8.41 (7.65) [a]	(3.41, 13.41)	5.50 (2.94) [a]	(3.58, 7.42)	9.57 (7.86) [a]	(4.43, 14.71)
CM	0.66 (0.74) [a]	(0.18, 1.14)	1.65 (2.43) [a]	(0.06, 3.24)	1.78 (1.77) [a]	(0.62, 2.94)
CPC	5.90 (3.72) [a]	(3.47, 8.33)	2.09 (1.40) [a]	(1.17, 3.01)	4.12 (2.61) [a]	(2.41, 5.83)

[ab] Means in the same row with different superscripts are significantly different ($P < 0.05$)

IL-1β was measured as a marker of both macrophage activity and inflammation (Table 5.6). For the PM diet, a significant decrease in abundance was observed for the 150 g kg^{-1} diet, compared with the control. A significant increase in IL-1β abundance was seen for the 300 g kg^{-1}

SBM diet with respect to the 150 g kg^{-1} diet, though neither was significantly different from the control diet. No other significant treatment effects were seen on the abundance of IL-1β.

Table 5.6 Mean (SD) abundance ($\times 10^1$ copies per 50 ng total RNA) of IL-1β mRNA in distal intestine of rainbow trout fed diets containing plant based ingredients at two levels of processing.

Ingredient [1]	Inclusion Level					
	0 g kg^{-1}		150 g kg^{-1}		300 g kg^{-1}	
	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI
PM	5.10 (5.09) [b]	(1.77, 8.43)	1.00 (1.06) [a]	(0.31, 1.69)	5.40 (3.60) [b]	(3.05, 7.75)
PPC	2.80 (1.98) [a]	(1.51, 4.09)	1.70 (2.38) [a]	(0.14, 3.26)	1.90 (0.73) [a]	(1.42, 2.38)
SBM	17.70 (10.18) [ab]	(11.05, 24.35)	10.20 (7.50) [a]	(5.30, 15.10)	44.30 (23.40) [b]	(29.01, 59.59)
SPC	7.80 (14.29) [a]	(-1.53, 17.13)	6.00 (3.17) [a]	(3.93, 8.07)	3.80 (2.12) [a]	(2.42, 5.18)
CM	1.30 (1.47) [a]	(0.34, 2.26)	2.30 (2.01) [a]	(0.99, 3.61)	2.40 (2.01) [a]	(1.09, 3.71)
CPC	3.10 (3.30) [a]	(0.94, 5.26)	3.50 (2.65) [a]	(1.77, 5.23)	4.10 (3.44) [a]	(1.85, 6.35)

[ab] Means in the same row with different superscripts are significantly different ($P < 0.05$)

IL-8 was evaluated as a marker of neutrophil infiltration to distal gut (Table 5.7). A significant decrease in abundance was seen for both the 150 g kg^{-1} and 300 g kg^{-1} PPC diets compared with the control diet, with the 300 g kg^{-1} diet being significantly higher than the 150 g kg^{-1} diet. A significant increase in abundance was seen for 300 g kg^{-1} SBM diet compared with the control. Finally, a significant increase in abundance was seen for the 300 g kg^{-1} CPC diet with respect to the 150 g kg^{-1} diet, though neither was significantly different from the control. No other significant treatment effects were seen on the abundance of IL-8.

Table 5.7 Mean (SD) abundance ($\times 10^2$ copies per 50 ng total RNA) of IL-8 mRNA in distal intestine of rainbow trout fed diets containing plant based ingredients at two levels of processing.

Ingredient [1]	Inclusion Level					
	0 g kg^{-1}		150 g kg^{-1}		300 g kg^{-1}	
	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI
PM	12.19 (3.65) [a]	(9.81, 14.57)	8.79 (4.56) [a]	(5.81, 11.77)	11.57 (2.40) [a]	(10.00, 13.14)
PPC	14.70 (3.28) [c]	(12.56, 16.84)	4.28 (1.98) [a]	(2.99, 5.57)	8.59 (1.80) [b]	(7.41, 9.77)
SBM	12.17 (3.08) [a]	(10.16, 14.18)	9.54 (2.97) [a]	(7.60, 11.48)	21.93 (8.68) [b]	(16.26, 27.60)
SPC	8.79 (4.84) [a]	(5.63, 11.95)	11.70 (8.29) [a]	(6.29, 17.11)	9.30 (3.90) [a]	(6.75, 11.85)
CM	3.17 (2.09) [a]	(1.80, 4.54)	5.09 (4.38) [a]	(2.23, 7.95)	3.53 (1.35) [a]	(2.65, 4.41)
CPC	9.98 (4.50) [ab]	(7.04, 12.92)	5.94 (3.59) [a]	(3.59, 8.29)	26.54 (27.29) [b]	(8.71, 44.37)

[ab] Means in the same row with different superscripts are significantly different ($P < 0.05$)

IL-10 was measured as an indicator of anti-inflammatory activity (Table 5.8). A significant increase in abundance was seen for the 300 g kg^{-1} PM diet compared with the 150 g kg^{-1} diet, though neither was significantly different from the control. A significant decrease in abundance was seen for the 150 g kg^{-1} PPC with respect to the control. No other significant treatment effects were seen on the abundance of IL-10.

Table 5.8 Mean (SD) abundance ($\times 10^2$ copies per 50 ng total RNA) of IL-10 mRNA in distal intestine of rainbow trout fed diets containing plant based ingredients at two levels of processing.

Ingredient [1]	Inclusion Level					
	0 g kg^{-1}		150 g kg^{-1}		300 g kg^{-1}	
	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI
PM	3.53 (1.30) [ab]	(2.68, 4.38)	1.83 (1.17) [a]	(1.07, 2.59)	6.37 (3.66) [b]	(3.98, 8.76)
PPC	4.52 (3.02) [b]	(2.55, 6.49)	1.66 (2.04) [a]	(0.33, 2.99)	4.64 (2.96) [b]	(2.70, 6.58)
SBM	2.69 (1.24) [a]	(1.88, 3.50)	1.59 (1.23) [a]	(0.79, 2.39)	1.54 (0.71) [a]	(1.08, 2.00)
SPC	1.21 (0.88) [a]	(0.64, 1.78)	1.32 (0.79) [a]	(0.80, 1.84)	2.18 (1.86) [a]	(0.96, 3.40)
CM	1.18 (0.84) [a]	(0.63, 1.73)	1.84 (1.84) [a]	(0.64, 3.04)	3.08 (2.88) [a]	(1.20, 4.96)
CPC	3.04 (2.04) [a]	(1.71, 4.37)	1.63 (0.93) [a]	(1.03, 2.23)	1.91 (0.93) [a]	(1.30, 2.52)

[ab] Means in the same row with different superscripts are significantly different ($P < 0.05$)

Caspase-3 activity was evaluated as an indicator of apoptosis in the distal intestine (Table 5.9). A significant decrease in activity was seen for both the 150 g kg^{-1} and 300 g kg^{-1} PPC diets, compared with the control diet. No other significant treatment effects were seen on the activity of caspase-3.

Table 5.9 Mean (SD) abundance (μmol g^{-1} of protein) of active caspase-3 in distal intestine of rainbow trout fed diets containing plant based ingredients at two levels of processing.

Ingredient [1]	Inclusion Level					
	0 g kg^{-1}		150 g kg^{-1}		300 g kg^{-1}	
	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI	$\bar{x}$ (SD)	95% CI
PM	2.56 (2.46) [a]	(0.95, 4.17)	1.01 (0.69) [a]	(0.56, 1.46)	2.85 (2.58) [a]	(1.16, 4.54)
PPC	1.58 (0.59) [a]	(1.19, 1.97)	0.50 (0.42) [b]	(0.22, 0.78)	0.30 (0.40) [b]	(0.04, 0.56)
SBM	0.88 (0.48) [a]	(0.57, 1.19)	1.93 (1.67) [a]	(0.84, 3.02)	5.60 (6.51) [a]	(1.35, 9.85)
SPC	3.42 (3.25) [a]	(1.29, 5.55)	5.36 (7.55) [a]	(0.43, 10.29)	1.37 (0.91) [a]	(0.78, 1.96)
CM	1.09 (0.68) [a]	(0.65, 1.53)	1.21 (0.51) [a]	(0.88, 1.54)	0.82 (0.42) [a]	(0.54, 1.10)
CPC	2.10 (0.85) [a]	(1.55, 2.65)	1.43 (0.88) [a]	(0.86, 2.00)	1.34 (1.19) [a]	(0.56, 2.12)

[ab] Means in the same row with different superscripts are significantly different ($P < 0.05$)

5.3 Correlation of Transcript Abundance with Treatment and Performance Variables

Pearson correlation coefficients were determined to assess the linear dependence between the abundance of individual genes and a range of performance and dietary parameters (Table 5.10). Performance data used for these calculations was reported previously by Collins *et al.* (2012). The abundance of mRNA transcripts associated with pro-inflammatory activity (IgM, IL-1β, IL-8) and cell turnover (PCNA, caspase-3) all showed significant positive correlations with one another and were not significantly correlated with the abundance of the anti-inflammatory marker IL-10. Additionally, significant negative correlations with specific growth rate (SGR) were observed for both IgM and IL-1β. Conversely, a significant positive correlation between IL-10 and SGR was seen. There were no significant correlations between average daily feed intake (ADFI) and any of the assayed markers (Table 5.10).

Table 5.10 Pairwise Pearson correlation coefficients among abundance of marker genes (log copies per 50 ng total RNA), caspase-3 activity (μmol g^{-1} of protein), SGR ((ln final weight (g) - ln initial weight (g)) / days × 100), and ADFI (feed intake (g) / fish / day).

Gene Target	Gene Target						
	PCNA	IgM	IL-1β	IL-8	IL-10	Caspase-3	SGR
IgM							
Pearson Correlation	0.770**						
Sig. (2-tailed)	0.000						
IL-1β							
Pearson Correlation	0.635**	0.723**					
Sig. (2-tailed)	0.000	0.000					
IL-8							
Pearson Correlation	0.600**	0.584**	0.602**				
Sig. (2-tailed)	0.000	0.000	0.000				
IL-10							
Pearson Correlation	0.134	-0.025	0.131	0.200			
Sig. (2-tailed)	0.335	0.859	0.345	0.147			
Caspase-3							
Pearson Correlation	0.393**	0.357**	0.398**	0.434**	-0.088		
Sig. (2-tailed)	0.003	0.008	0.003	0.001	0.527		
SGR							
Pearson Correlation	-0.097	-0.340*	-0.325*	-0.037	0.281*	-0.115	
Sig. (2-tailed)	0.484	0.012	0.016	0.789	0.040	0.406	
ADFI							
Pearson Correlation	0.003	0.014	0.034	0.141	0.022	0.068	0.212
Sig. (2-tailed)	0.986	0.924	0.811	0.323	0.880	0.636	0.135

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Correlations of the inclusion level of plant-based protein sources with selected immune and performance markers are shown in Table 5.11. Pea meal inclusion was positively correlated with both IL-10 and SGR. Pea protein concentrate inclusion was negatively correlated with caspase-3 activity and positively correlated with SGR. Non-significant trends toward negative correlation with the abundance of IL-1β and positive correlation with ADFI were also seen for the inclusion of PPC. Soybean meal inclusion was positively correlated with all assayed proinflammatory and immune markers, with the exception of IL-10, and was negatively correlated with SGR. The inclusion of soy protein concentrate was not significantly correlated with any of the selected markers, but tended to be positively correlated with the abundance of IgM. Canola meal inclusion was negatively correlated with PCNA and IL-8 abundance and also tended to be negatively correlated with the abundance of IgM. The inclusion of canola protein concentrate was not significantly correlated with any of the inflammatory or immune markers, but was negatively correlated with SGR.

Table 5.11 Pairwise Pearson correlation coefficients among inclusion of plant-based protein sources (g kg^{-1}), abundance of marker genes (log copies per 50 ng total RNA), caspase-3 activity (µmol g^{-1} of protein), SGR ((ln final weight (g) - ln initial weight (g)) / days × 100), and ADFI (feed intake (g) / fish / day).

Target	Ingredient					
	PM	PPC	SBM	SPC	CM	CPC
PCNA						
Pearson Correlation	0.059	-0.081	0.357**	0.198	-0.319*	-0.090
Sig. (2-tailed)	0.672	0.561	0.008	0.151	0.019	0.518
IgM						
Pearson Correlation	-0.176	-0.176	0.519**	0.232	-0.266	-0.056
Sig. (2-tailed)	0.203	0.203	0.000	0.091	0.052	0.687
IL-1β						
Pearson Correlation	-0.049	-0.229	0.612**	0.016	-0.175	-0.028
Sig. (2-tailed)	0.723	0.096	0.000	0.909	0.207	0.840
IL-8						
Pearson Correlation	0.107	-0.126	0.341*	0.072	-0.441**	0.176
Sig. (2-tailed)	0.441	0.366	0.012	0.606	0.001	0.203
IL-10						
Pearson Correlation	0.340*	0.152	-0.215	-0.107	0.087	-0.093
Sig. (2-tailed)	0.012	0.271	0.118	0.442	0.532	0.506
Caspase-3						
Pearson Correlation	0.106	-0.432**	0.335*	0.135	-0.213	-0.071
Sig. (2-tailed)	0.444	0.001	0.013	0.329	0.122	0.612
SGR						
Pearson Correlation	0.408**	0.366**	-0.359**	-0.095	-0.207	-0.399**
Sig. (2-tailed)	0.002	0.007	0.008	0.497	0.132	0.003
ADFI						
Pearson Correlation	0.017	0.234	0.053	-0.069	-0.631**	0.110
Sig. (2-tailed)	0.904	0.098	0.711	0.629	0.000	0.440

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

Correlations between the immune / performance markers and the inclusion level of feed ingredients common among many of the experimental diets are shown in Table 5.12. Fish oil inclusion was positively correlated with both IL-10 abundance and SGR. Fish meal inclusion was negatively correlated with the abundance of PCNA and IgM, while tending to be negatively correlated with IL-8 abundance. The inclusion of corn gluten meal was positively correlated with

IL-10 abundance and SGR. Alpha-cellulose inclusion was negatively correlated with the abundance of both PCNA and IL-8. No statistically significant correlations with the immune and performance markers were found for the inclusion of meat and bone meal.

Table 5.12 Pairwise Pearson correlation coefficients among inclusion of experimental ingredients (g kg^{-1}), abundance of marker genes (log copies per 50 ng total RNA), caspase-3 activity (µmol g^{-1} of protein), SGR ((ln final weight (g) - ln initial weight (g)) / days × 100), and ADFI (feed intake (g) / fish / day).

Target	Ingredient				
	Fish Oil[a]	Fish Meal[b]	CGM[c]	MBM[d]	Alpha-cellulose[e]
PCNA					
Pearson Correlation	0.206	-0.362**	0.050	-0.088	-0.288*
Sig. (2-tailed)	0.136	0.007	0.721	0.526	0.035
IgM					
Pearson Correlation	-0.017	-0.377**	-0.222	-0.019	-0.023
Sig. (2-tailed)	0.902	0.005	0.107	0.891	0.867
IL-1β					
Pearson Correlation	0.051	-0.200	-0.146	-0.102	0.165
Sig. (2-tailed)	0.713	0.147	0.293	0.463	0.234
IL-8					
Pearson Correlation	0.199	-0.252	0.064	-0.125	-0.325*
Sig. (2-tailed)	0.150	0.066	0.643	0.366	0.016
IL-10					
Pearson Correlation	0.315*	0.078	0.423**	-0.221	-0.171
Sig. (2-tailed)	0.020	0.574	0.001	0.108	0.216
Caspase-3					
Pearson Correlation	0.180	-0.069	-0.169	0.147	-0.177
Sig. (2-tailed)	0.192	0.619	0.223	0.288	0.201
SGR					
Pearson Correlation	0.478**	-0.076	0.632**	0.086	-0.379**
Sig. (2-tailed)	0.000	0.582	0.000	0.538	0.005
ADFI					
Pearson Correlation	0.073	-0.225	0.171	0.135	-0.336*
Sig. (2-tailed)	0.610	0.112	0.231	0.346	0.016

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).
[a] Danish Fish Oil; FF of Denmark, Skagen, Denmark.
[b] Nova Scotia herring meal; Shur-Gain Aquaculture, Truro, NS, Canada.
[c] Corn Gluten Meal
[d] Meat and bone meal; Saskatoon Processing Co., Saskatoon, SK, Canada.
[e] Solka-floc®, 200 FCC; International fibre corporation, North Tonawanda, NY, USA.

Finally, correlations between immune and performance markers in relation to the abundance of putative ANFs found in the experimental diets are shown in Table 5.13. Starch content was positively correlated with both IL-10 abundance and SGR. Phytate content was negatively correlated with the abundance IL-8 and the activity of caspase-3, while tending to be negatively correlated with both IgM abundance and ADFI. Glucosinolates were negatively correlated with the abundance of PCNA, IgM, and IL-8 as well as both SGR and ADFI. Tannins were not significantly correlated with any of the immune or performance markers, but tended to be negatively correlated with SGR and ADFI. Saponin content was negatively correlated with SGR and ADFI, while tending to be positively correlated with the abundance of IL-8. Isoflavones were negatively correlated with SGR and positively correlated with the abundance of PCNA, IgM, IL-1β, IL-8, and the activity of caspase-3. The abundance of insoluble NSP was negatively correlated with SGR and ADFI. No statistically significant correlations with the immune and performance markers were found for soluble NSP content.

Table 5.13 Pairwise Pearson correlation coefficients among putative antinutritional factor content (g kg^{-1}), abundance of marker genes (log copies per 50 ng total RNA), caspase-3 activity (µmol g^{-1} of protein), SGR ((ln final weight (g) - ln initial weight (g)) / days × 100), and ADFI (feed intake (g) / fish / day).

Target	Dietary Component							
	Starch	Phytate	Glucosinolates	Tannins	Saponins	Isoflavones	Soluble NSP	Insoluble NSP
PCNA								
Pearson Correlation	0.033	-0.205	-0.336*	-0.015	0.106	0.359**	0.046	-0.031
Sig. (2-tailed)	0.811	0.136	0.013	0.912	0.447	0.008	0.742	0.825
IgM								
Pearson Correlation	-0.217	-0.237	-0.277*	0.019	0.208	0.520**	-0.025	0.022
Sig. (2-tailed)	0.116	0.084	0.043	0.889	0.131	0.000	0.856	0.873
IL-1β								
Pearson Correlation	-0.128	-0.183	-0.180	0.096	0.244	0.612**	0.004	0.058
Sig. (2-tailed)	0.358	0.186	0.192	0.491	0.075	0.000	0.974	0.676
IL-8								
Pearson Correlation	0.043	-0.343*	-0.412**	-0.059	0.122	0.342*	0.022	-0.062
Sig. (2-tailed)	0.760	0.011	0.002	0.669	0.378	0.011	0.876	0.657
IL-10								
Pearson Correlation	0.378**	0.186	0.071	0.062	-0.096	-0.215	0.163	0.019
Sig. (2-tailed)	0.005	0.179	0.610	0.657	0.490	0.119	0.240	0.890
Caspase-3								
Pearson Correlation	-0.126	-0.401**	-0.227	-0.150	0.144	0.336*	-0.221	-0.104
Sig. (2-tailed)	0.362	0.003	0.100	0.278	0.297	0.013	0.108	0.455
SGR								
Pearson Correlation	0.530**	0.101	-0.278*	-0.268	-0.540**	-0.358**	0.039	-0.374**
Sig. (2-tailed)	0.000	0.466	0.042	0.050	0.000	0.008	0.780	0.005
ADFI								
Pearson Correlation	0.129	-0.236	-0.606**	-0.245	-0.279*	0.053	0.002	-0.316*
Sig. (2-tailed)	0.366	0.096	0.000	0.083	0.048	0.711	0.986	0.024

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

5.4 Histological Examination of Distal Gut Tissues

Microscopic examination of fixed distal gut cross sections after haematoxylin and eosin staining indicated significant physical damage to villi, including: sheared villus tips, fracturing of enterocytes, and separation of enterocytes from the lamina propria (Figure 5.1). These features were indicative of technical problems associated with tissue harvesting, fixing, and processing.

Attempts to improve collection and processing procedures, including minimized physical handling and a range of modifications to the fixation protocol did not markedly improve cross section quality. The poor quality of preparations prevented accurate measurement of mucosal parameters such as villus length, width of the lamina propria, presence of supranuclear vacuoles in enterocytes, and goblet cell numbers. The micrographs presented in Figure 5.1 are representative of the average quality obtained for the 0 g kg^{-1} control diet and the 300 g kg^{-1} maximum inclusion diet for each meal ingredient.

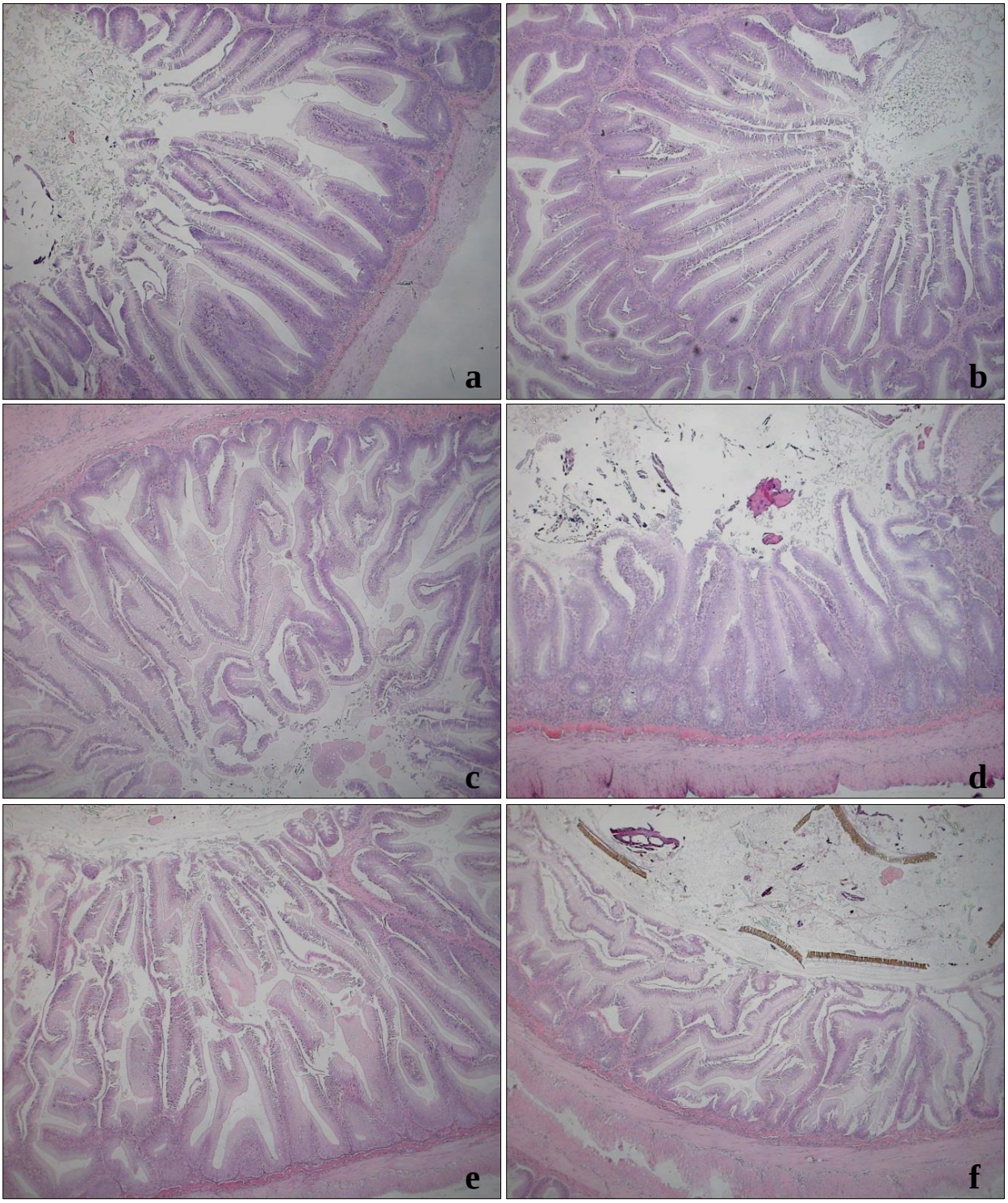

Figure 5.1 Representative distal gut cross sections stained with hematoxylin and eosin from the 0% PM (a), 30% PM (b), 0% SBM (c), 30% SBM (d), 0% CM (e), and 30% CM (f) experimental diets at 50× magnification.

6.0 DISCUSSION

In order to monitor physiological changes in the gastrointestinal tract of rainbow trout fed different plant-based protein sources at two distinct processing levels it was necessary, due to facility capacity, to conduct six separate feeding trials in succession. To minimize impact of the successive studies, the same group of fish was used for all six experiments. Additionally, each experiment included an independent control group with zero test ingredient inclusion. Furthermore, the trials were scheduled such that the two processing levels of each feed ingredient were tested either simultaneously or in short succession. All six trials were completed within a one year period. Mean body weight of the fish was 235.2 ± 17.1 g at the beginning of the first trial and 824.6 ± 82.8 g at the beginning of the sixth trial. As a result, statistical comparisons were limited to within protein source and processing level. Since the Pearson correlations were based on pairwise comparisons of data points within experiments, correlation analysis was assessed based on data from all six experiments combining protein sources and processing levels. This approach permitted the identification of interesting associations, which offer insight into physiological mechanisms limiting ingredient inclusion and the antinutritional factors which may be causative.

Major challenges faced in the analysis of this study were the high level of variation in transcript abundance and the failure to identify a suitable housekeeping gene for internal normalization of qPCR data (de Jonge *et al.*, 2007). Fish-to-fish variation in transcript abundance was characterized by coefficient of variation values well over 10%, despite the removal of outlier values based on established statistical procedures. This variation could not be associated with any technical error in sample collection or analysis and was evident independent of reagent lot or technical staff conducting the analysis. Although fish chosen for tissue collection were selected as representing median within-tank body size, significant variations in fish body weight were evident. Differences in the range of 2-fold from lightest to heaviest within trials may have contributed to the observed variations in transcript abundance; however, no correlation between body weight and transcript abundance was evident for housekeeping genes (data not shown).

Furthermore, mRNA transcript abundance was frequently lowest in the 150 g kg^{-1} diet, regardless of protein source or processing level. The diets were formulated to be isoenergetic and isonitrogenous, based on digestible nutrients, while keeping fish meal at a near constant level,

within the limitations of formulation. This is in contrast to most other studies utilizing plant-based protein sources where fish meal was progressively replaced by the test ingredient (Gomes *et al.*, 1993; Stickney *et al.*, 1996; Carter and Hauler, 2000). In the current study, corn gluten meal and meat and bone meal were displaced by test ingredients. These ingredients are reported to lack both beneficial and detrimental properties, making them ideal for substitution (Alexis *et al.*, 1985; Bureau *et al.*, 2000; Hardy, 2000; Yu, 2004). Because the trials were conducted in succession and tank assignment to each treatment was randomized for each trial, data for the 150 g kg^{-1} treatment groups represent essentially unique tanks for each protein source. Furthermore, this intermediate inclusion level was prepared by blending of the 0 and 300 g kg^{-1} diets (Collins *et al.* 2012), making it unlikely that the results for the 150 g kg^{-1} group reflect a technical error in diet preparation. The source of observed variations in transcript abundance at this inclusion level, which were also apparent in the performance data reported by Collins *et al.* (2012), is not readily explainable. Finally, although a statistical comparison was not appropriate, review of the transcript abundance values for candidate housekeeping genes in the 0 g kg^{-1} control diets indicated marked variation. Variation did not correlate with the order in which experiments were conducted, suggesting that differences could not be directly attributable to seasonal environment, fish age, or body weight. A change in ingredient sourcing and subsequent modification in formulation occurred between the SBM and SPC trials, but again the pattern of transcript abundance in housekeeping gene candidates did not support this as a contributing factor. One factor which may have contributed to both high fish-to-fish variation and the variation among control diets was the management of fish prior to sample collection. The day prior to collection, fish were removed from their tanks by net and transferred to a large tub to be weighed for determination of SGR and other performance parameters. It is possible that the high stress level induced by handling in such close proximity to sample collections contributed to the observed variation in transcript abundance. Several studies have documented the significant and prolonged effects of stress, including handling stress, on the abundance of a large number of genes in varied tissues of rainbow trout, supporting this possibility (Krasnov *et al*., 2005; Momoda *et al*., 2007; Pemmasania *et al*., 2011).

Based on these findings, qPCR data for this study is reported as transcript copy number per 50 ng of total RNA rather than transcript copy number per copy of an internal control gene. Though this is not the most common method of normalization, in some cases this approach has

been shown to be more reliable than housekeeper-based normalization methods (Bustin, 2000; Bustin, 2002).

Using performance data from the same experiment as reported here, Collins *et al.* (2012) performed both linear and quadratic regression analysis of SGR, ADFI, feed conversion ratio (FCR), and protein efficiency ratio (PER) with inclusion level for each protein source and level of processing. Briefly, there were no significant linear or quadratic regressions found for the inclusion level of PM, SPC or CPC. Inclusion level of PPC showed a positive linear relationship with ADFI. Inclusion level of SBM had a negative quadratic relationship with both SGR and FCR, while its negative relationship with PER was significant for both linear and quadratic regressions. Inclusion level of CM also showed a strong negative association with SGR, FCR, and PER. Based on these findings, CM was least tolerated as a protein source followed by SBM, and PM. Further processing of these ingredients apparently removed any negative effects on performance; indeed, PPC may have positively affected performance. These results are in general agreement with previous reports assessing the nutritional value and antinutritional properties of these ingredients (see Collins *et al.* 2012).

The results of the univariate GLM analysis comparing inclusion level for each ingredient are difficult to interpret. Very high standard error values were present, as described above, which were not easily explained. Despite the strong negative relationship observed between performance parameters and the inclusion level of CM both in this study (Collins *et al*., 2012) and elsewhere (Alami-Durante *et al.*, 2010), no significant effect on transcript abundance in distal gut proinflammatory markers was observed when CM was fed at either 150 or 300 g kg^{-1}. The level of inclusion of SBM was also negatively correlated with growth performance by Collins *et al*. (2012) but again, no relationship between level of inclusion of SBM and abundance of proinflammatory markers PCNA, IgM, or IL-1β was evident. In fact, abundance of these markers was commonly lowest when SBM was included at 150 g kg^{-1} in the diet. In contrast to these observations, others have reported increased abundance of proinflammatory markers when SBM was included at similar levels in Atlantic salmon diets associated with reduced performance (Bakke-McKellep *et al.*, 2000; Krogdahl *et al*., 2000). Only the increased abundance of the neutrophil chemokine IL-8 and the B cell marker IgM, at the 300 g kg^{-1} inclusion level, were consistent with a proinflammatory response to SBM in the present study. Consistent with the limited response of proinflammatory transcript abundance observed at high

inclusion levels of CM and SBM, there was also very little impact with inclusion of the associated protein concentrates. The SPC and CPC trials showed almost no significant treatment effects, with the exception of increased IL-8 abundance for fish fed CPC at 300 g kg^{-1} inclusion. This is not an unexpected result, since ANF levels are very low in both SPC and CPC in comparison with SBM and CM (Collins *et al*., 2013). Consequently, their expected impact on both performance and intestinal physiology was very low. Indeed, no negative impacts of SPC inclusion were found up to our maximum inclusion level of 300 g kg^{-1}, which is in agreement with expectations, based on existing research (Bureau *et al*., 1998; Barrows *et al*., 2007). Previous studies of CPC inclusion have had mixed results (Stickney *et al.*, 1996; Thiessen *et al.*, 2004; Drew *et al.*, 2007), possibly due to variances in the methods used to produce the protein concentrate ingredient, including the use of phytase-treatment by some researchers (Collins *et al.*, 2012).

Examination of differences in transcript abundance with inclusion of PM and PPC suggested that these ingredients may have reduced inflammatory responses. PM inclusion did not increase abundance of proinflammatory markers but appeared to increase abundance of the anti-inflammatory cytokine IL-10 at the 300 g kg^{-1} level. Prior studies on the inclusion of PM in rainbow trout diets have shown both positive and negative effects on performance (Thiessen *et al.*, 2003; Drew *et al.*, 2005). No significant effects of PM inclusion were observed on the regression analysis of performance parameters for this study (Collins *et al*., 2012).

Results in fish fed PPC also supported a possible anti-inflammatory response. Abundance of IL-8 and the activity of caspase-3 were both reduced in the 150 g kg^{-1} and 300 g kg^{-1} treatment groups, relative to the control, although a dose response relationship was not evident. The abundance of IL-10 was also reduced for the 150 g kg^{-1} treatment group but the 300 g kg^{-1} group was not significantly different from the control. A significant decrease in the abundance of IL-8 and the activity of caspase-3 could indicate a possible benefit to the inclusion of PPC in the diet, resulting in reduced inflammatory and apoptotic activities. Other studies have shown generally neutral effects of PPC on performance (Øverland *et al.*, 2009; Gao *et al.*, 2011). Very high inclusion levels, above our maximum, have been shown to have detrimental impacts on intestinal health and growth (Penn *et al.*, 2011). No reports were found in which parameters associated with intestinal immune response were measured following dietary inclusion of PPC in carnivorous fish.

In an attempt to account for significant fish-to-fish variation in size and high variation in treatment means, based on inclusion level, Pearson correlation coefficients were determined between transcript abundance values, performance parameters, protein source, inclusion level, and dietary ANF concentration (Collins *et al*., 2013). It was anticipated that pairwise correlation analysis would reveal associations warranting further investigation into causal relationships. Significant positive correlations were observed between all combinations of proinflammatory markers (PCNA, IgM, IL-1β, IL-8, and caspase-3), independent of dietary treatment. This is consistent with a coordinated inflammatory cascade following proinflammatory cytokine secretion, including: B cell and neutrophil activation, increased leukocyte proliferation, and increased enterocyte replacement (Kumar *et al*., 2010; Murray and Wynn, 2011; Rieger and Barreda, 2011; Rombout *et al*., 2011). Abundance of IL-1β and IgM were negatively correlated with SGR, consistent with observations in avian species and mammals, correlating gut inflammation, absorptive tissue damage, and nutrition partitioning away from growth (Wolf *et al*., 1996; Johnson, 1997; Klasing and Korver, 1997). Although systemic proinflammatory responses have been shown to suppress appetite (Aubert *et al*., 1995; Langhans and Hrupka, 2003), no correlations between proinflammatory transcript abundance in distal gut of trout and ADFI were observed here.

None of the proinflammatory markers were significantly correlated with the abundance of the anti-inflammatory marker IL-10. That being said, none of the proinflammatory genes assessed are known to be important regulators of IL-10 abundance (Saraiva and O'Garra, 2010; Ouyang *et al.*, 2011). The significant positive correlation of IL-10 with SGR suggests that immunoregulation by IL-10 may, in some cases, support growth performance.

Pairwise correlations of transcript abundance and performance parameters with inclusion levels of the six alternative protein sources were in general agreement with treatment differences observed by the univariate GLM analysis. Dietary SBM inclusion was positively correlated with the abundance of all proinflammatory markers and negatively correlated with SGR, consistent with previous observations of gut inflammation associated with this ingredient (Burrells *et al.*, 1999; Bakke-McKellep *et al.*, 2000; Buttle *et al.*, 2001; Krogdahl *et al.*, 2003; Sanden *et al.*, 2005). Although CM was the least tolerated protein source by fish in this study, two proinflammatory markers, IL-8 and PCNA, were surprisingly negatively correlated with its level of inclusion. Curiously, a strong negative correlation was observed between CM inclusion and

ADFI, but not SGR. Analysis of performance data from this trial by Collins *et al*. (2012) did identify significant regressions for SGR and other performance parameters at predicted inclusion levels as low as 2 - 4 g kg^{-1}. The negative correlation of CM with IL-8 and PCNA, potentially indicating downregulation, suggests that factors other than gut inflammation are influencing the poor performance associated with its inclusion in aquaculture diets. Palatability can affect ADFI and indeed trout may be particularly sensitive to compounds such as phenols and glucosinolates in CM (Higgs *et al*., 1995). After further processing of SBM and CM, to SPC and CPC respectively, no correlations between the inclusion level of these ingredients and any pro- or anti-inflammatory markers were observed. This result suggests that the causative agent(s), responsible for upregulation (or downregulation) of inflammatory markers and/or palatability changes, were either significantly reduced or eliminated by processing of the ingredients to a purified protein concentrate.

Correlations between gene markers and dietary inclusion of PM and PPC again showed contrasting results to SBM, similar to treatment comparisons by the univariate GLM analysis of variance. Both ingredients were positively correlated with SGR. In the case of PM, a positive correlation with IL-10 was also observed, whereas PPC was associated with reduced caspase-3 activity in the distal gut. These observations are consistent with ingredients that are well tolerated by the intestinal tract. Indeed, it is tempting to speculate that, in the case of PM, further processing to PPC resulted in the loss or partial loss of an anti-inflammatory factor associated with induction of IL-10.

Pearson correlations were also examined between tissue markers and performance parameters with the most common feed ingredients across all six experimental diets. With the exception of corn gluten meal (CGM), all ingredients examined were present in all six diets. Due to the fact that the experimental diets were formulated to meet the nutritional requirements for growth, rather than to simply replace fish meal, there was considerable variation in the inclusion rates of several ingredients. In some cases, inclusion of ingredients to nutritionally balance diets was primarily driven by the composition of the test ingredient. This situation confounds correlations between ingredients and tissue markers with the tested protein sources. For example, CGM was present only in the SBM, PM, and PPC diets; however, its content was considerably higher in the pea-based diets. As a result, any correlations with CGM are likely to be confounded with inclusion of PM and PPC. Such is probably the case where CGM inclusion was positively

correlated with SGR and IL-10 abundance, similar to PM. On the other hand, there is some evidence that the L-alanyl-L-glutamine (Ala-Gln) dipeptide, abundant in CGM, may limit pathogen proliferation and/or provide supplemental glutamine to distal gut enterocytes (Fuentes *et al.*, 2004; Annett Christianson, 2011) providing a health benefit.

Dietary inclusion of fish oil was also positively correlated with SGR and IL-10 abundance. There is some evidence in the literature of a connection between dietary fish oil inclusion and anti-inflammatory activity, but the effect does not appear to be mediated by an increase in IL-10 production. Rather, it has been found that omega-3 and omega-6 fatty acids in fish oil may significantly reduce the production and biological activity of prostaglandins, which would be expected to result in reduced abundance of IL-10 (Shinomiya, *et al.*, 2001; Smith, W.L., 2006) rather than the apparent increase that was observed. The concentration of fish oil was lowest in the canola-based diets and similar between the soybean and pea-based diets, indicating that its association with increased abundance of IL-10 may be driven by the PM diet.

Fish meal inclusion was negatively correlated with the abundance of PCNA and IgM, and displayed a trend toward a negative correlation with IL-8 abundance. There is limited evidence that fish meal may positively affect immune status and gut health. Dietary fish meal inclusion has been reported to increase abundance of Gram positive bacteria and increase microbial diversity in distal gut of teleosts (Ringø *et al.*, 2006; Mansfield *et al.*, 2010; Desai *et al.*, 2012) which could mediate beneficial effects on the gut mucosa.

Dietary inclusion of α-cellulose showed negative correlations with two proinflammatory markers, IL-8 and PCNA, as well as with SGR and ADFI. This was surprising, as the purified α-cellulose product used is metabolically inert and is therefore highly unlikely to have a direct impact on immune markers. The PM diet did not contain α-cellulose; however, all other diets varied in content by about two fold, with the highest concentration found in the SPC diet and the lowest concentration in the CM diet. PM was not correlated with proinflammatory markers and likely did not confound the observation for α-cellulose. The observed correlation pattern most closely matched CM but, given that α-cellulose inclusion in the CM diets was the lowest tested, it is unlikely that CM was a confounding factor.

Collins *et al*. (2013) analyzed all experimental diets for content of a variety of putative antinutritional factors which may have contributed to the tissue marker responses observed. Because multiple ANFs are present in several ingredients, correlations between ANF content and

tissue markers could provide some insight into possible cause-effect relationships. Nevertheless, as for the correlations with non-test ingredients, correlations for ANF must also be interpreted carefully to avoid confounding influences.

Dietary inclusion of starch showed positive correlations with abundance of IL-10 and with SGR. This observation is initially surprising as carnivorous teleosts are not well adapted to consuming high levels of starch in their diet and express comparatively low levels of carbohydrase enzymes (Buddington *et al*., 1997; Hidalgo *et al*., 1999; Krogdahl *et al*. 2005). High starch diets are known to result in poor utilization of dietary energy, decreased macronutrient digestibility, and reduced growth of rainbow trout (Hilton *et al.*, 1983; Thiessen *et al.*, 2003). Starch would, therefore, not be expected to support growth or elicit an anti-inflammatory response. Although starch is present in all tested diets, it is ten times more abundant in the PM diet. It is likely that this abundance accounts for the common correlation profile of starch with PM. Indeed, it is curious that fish fed PM performed well considering the abundance of poorly tolerated starch.

Dietary inclusion of both phytate and glucosinolates showed unexpected negative correlations with inflammatory markers. Phytate was negatively correlated with IL-8 abundance and caspase-3 activity, while tending toward negative correlation with the abundance of IgM and with ADFI. Glucosinolate inclusion was negatively correlated with three inflammatory markers, PCNA, IgM, and IL-8, in addition to SGR and ADFI. Based on their respective properties, the negative correlation of these compounds with inflammatory markers was unexpected. The major antinutritional properties of phytic acid are decreased mineral availability (Duffus and Duffus, 1991; Francis *et al*., 2001), decreased protein digestion (Cosgrove, 1966; Caldwell, 1992; Morales *et al*., 2011), and increased endogenous loss (Cowieson *et al*., 2004; Bedford and Cowieson, 2009). Although CPC does not contain phytic acid, due to the fact that the tested ingredient was dephytinized, it is found in the five remaining ingredients with concentrations ranging from 4.8 - 11.6 g kg^{-1}. Of the six experimental diets, the CM diet had the highest concentration (Collins *et al.*, 2013). In studies where phytic acid was fed directly to rainbow trout, reported effects included: decreased digestibility of crude protein, decreased growth rate / body condition, and declines in feed conversion ratio (Spinelli *et al*., 1983; Chowdhury *et al*., 2012; Chowdhury *et al*., 2014)

In the case of glucosinolates, the major antinutritional properties are suppressed thyroid function / growth performance (van Etten and Tookey, 1979; Underhill, 1980; Larsen, 1981; Lanno and Dixon, 1996) and irritation of the intestinal epithelium (Gaul, 1964; Fuller and McClintock, 1986; Brown, 2013). Of the six experimental ingredients, only the CM diet contained an appreciable level of glucosinolates (Collins *et al.*, 2013). When compared to the other correlations, the profiles for both phytate and glucosinolates most closely resemble the correlations for the CM diet. This should not be surprising, since this diet represents the maximum inclusion level for both of these ingredients, particularly in the case of glucosinolates, which has the potential to confound the analysis. It remains unclear why the poorly tolerated CM treatment, in terms of growth performance, is negatively associated with inflammatory markers. Regardless, as noted above, the mechanisms driving poor tolerance of CM clearly differ from the proinflammatory mechanisms observed with SBM.

Dietary inclusion of tannins was not significantly correlated with any of the performance or immune markers but tended to have a weak negative correlation with both SGR and ADFI. The antinutritional properties of tannins are primarily attributable to reductions in the bioavailability of protein, starch, and minerals. Tannin content also has the potential to reduce palatability, having a negative impact on feed intake (Liener, 1989; Francis *et al.*, 2001; Cannas, 2013). The tannin content of the experimental diets ranged two-fold from 1.5 - 3.2 g kg^{-1}, lowest in the PM diet and highest in the CM diet (Collins *et al.*, 2013). As a result, the negative effects of CM inclusion on SGR and ADFI may be driving this correlation. Interestingly, unlike some of the other ANFs abundant in CM, no negative correlation with proinflammatory markers is observed for tannins. This suggests the possibility that a tannin-mediated effect on palatability could be the main antinutritional driver for CM.

Dietary inclusion of saponins showed a negative correlation with SGR and ADFI, while tending toward a positive correlation with the abundance of IL-1β. As an antinutrient, saponins have several modes of action. Negative impacts due to dietary inclusion can include: decreased palatability; decreased cholesterol absorption, due to precipitation from digesta; permeabilization of cell membranes, resulting in cell lysis or vulnerability to pathogen ingress; reduced lipid digestion / absorption through sequestration of bile acids (Milgate and Roberts, 1995; Francis *et al.*, 2001; Francis *et al.*, 2002). Alcohol extracted soy saponins have been shown to cause damage to distal intestinal mucosa in several related species, including: Atlantic salmon,

Chinook salmon, and rainbow trout (Bureau *et al.*, 1998; Knudsen *et al.*, 2008). Further, a saponin-mediated increase in gut permeability has been implicated as a crucial factor in the onset of soybean meal-induced enteritis in Atlantic salmon (Bureau *et al.*, 1998; Knudsen *et al.*, 2008; Sørensen *et al.*, 2011). Four of the six experimental diets contained saponins, ranging from 0.5-1.6 g kg^{-1}, with the PPC diet having the highest concentration. The SPC and CPC diets did not contain a detectable amount of saponins (Collins *et al.*, 2013) nor does any single ingredient or antinutritional factor vary directly or indirectly with saponin content that might obviously confound the observed correlations. Interestingly, significant correlations observed for the PPC diet are opposite to those observed for dietary saponin abundance. It is possible that, like tannins, saponins role in reducing palatability may be a dominant factor contributing to the poor tolerance of CM, without contributing to inflammation.

Dietary isoflavone content demonstrated consistent positive correlation with proinflammatory markers as well as a negative correlation with SGR. Due to their structure, isoflavones are capable of acting as ligands for estrogen receptors and peroxisome proliferator-activated receptors (PPARs) (Medjakovic *et al.*, 2010; Penumetcha and Santanam, 2012). Their overall impact in teleost fish is not fully understood, but they have the potential to negatively affect both estrogen metabolism and growth performance while conversely having the potential to be beneficial for control of the inflammatory response, through PPAR-mediated attenuation, in contrast to the correlations observed (Clark, 2002; Arnold and König, 2006; Mazzon *et al.*, 2009; Wang *et al.*, 2011; Di Paola, 2011). Unfortunately, since isoflavones are only present in significant quantity in the SBM diets, the observed correlations likely reflect confounding effects with other SBM factors rather than a direct isoflavone effect.

Inclusion of insoluble NSPs showed negative correlations with both SGR and ADFI. By nature of their insolubility, these compounds remain suspended in digesta and do not have a significant effect on viscosity, though they can add bulk and have a laxative effect in sufficient quantities (Smits and Annison, 1996; Amirkolaie *et al.*, 2005). High levels of insoluble fibre can also have a negative effect on the stability of feed pellets in water, potentially affecting feed intake (FAO, 1980; Lim *et al.*, 1997). Additionally, indigestible dietary fractions can have a significant impact on the diversity and composition of the intestinal microbiome, acting as selective fermentation substrates. The literature reveals conflicting results for the impact of insoluble NSPs on feed intake, nutrient digestibility, and growth of teleost fish (Amirkolaie *et*

al., 2005; Glencross, B., 2009; Glencross *et al.*, 2012). This discrepancy likely arises from a combination of factors, including varied plant sources and perhaps a general oversimplification NSPs as a class. Compounds considered to be NSPs include several distinct classes of polysaccharides with differing effects, which could prove problematic when attempting to assess them collectively. It is clear that further research into this area is required in order to fully understand the impact of these fibre fractions on the gut health and performance of rainbow trout (Glencross *et al.*, 2012; Collins, S.A., 2014). Insoluble NSPs ranged more than two-fold from 3.4 - 8.5 g kg^{-1} across all test diets. The highest concentrations were found in the CM and CPC diets and the lowest in the PM diet (Collins *et al.*, 2013). The significant values seen in the Pearson correlation profile align with corresponding values in the canola-based diets, which had the highest concentrations of insoluble NSPs. More in-depth analysis by Collins (2014) suggests that insoluble NSPs did not have a direct effect on growth or feed intake. Rather, insoluble NSPs may have an indirect mode of action, interacting with other ANFs in the diet, resulting in the observed correlations (Collins, 2014).

Soluble NSPs were also investigated as putative ANFs. Dietary content ranged two-fold from 1.6 - 3.3 g kg^{-1}, with the highest levels found in the PPC diet and the lowest in the CPC diets (Collins *et al.*, 2013). Soluble high molecular weight NSPs, such as glucans and arabinoxylans, have the ability to increase the viscosity of digesta, delay gastric emptying, decrease passage rate, chelate cholesterol and divalent cationic minerals (Fe^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}), and cause an overall decrease in nutrient digestibility that have been documented in other monogastric species (Almirall *et al.*, 1995; Choct *et al.*, 1996; Amirkolaie *et al.*, 2005). Despite these potentially detrimental effects, results of the univariate GLM analysis, Pearson correlations, and performance data analysis did not reveal any significant treatment effects for inclusion of soluble NSP (Collins *et al.*, 2013).

As noted above, Pearson correlation coefficients were determined between selected parameters as a means of uncovering diet composition and host response relationships not apparent using the univariate GLM procedure. Additionally, this method allowed for incorporation of diet characteristics and fish performance parameters, reported previously by Collins *et al.* (2012, 2013), into the final analysis. While this approach did identify potential relationships between diet composition and host responses, a more rigorous multivariate approach using principal component analysis or structural equation modelling is warranted.

The results of the histological examination of distal gut cross sections were not as expected. The quality of our slides was very inconsistent and generally poor over the course of the experiment, despite several attempts to elucidate the cause and to make refinements to our collection and processing protocols. Our initial protocol for sample collection was based on established methods used successfully in our lab for collection of histological specimens from pigs. It could also be compared to the methods employed by Baeverfjord and Krogdahl (1996), in Atlantic salmon, though with less handling of the samples prior to fixation.

The initial protocol was to cut samples, approximately 1.5 cm in length, from an undisturbed region of the distal gut directly into 10% neutral buffered formalin. The samples were allowed to fix for 24 - 48 hours and were then removed from the formalin fixative for trimming. The fixed samples were trimmed to approximately 4 mm in length, carefully rinsed of excess digesta, and placed into tissue cassettes. The tissue cassettes were then immersed in 70% EtOH and delivered to PDS for sectioning and staining. The resulting slides varied widely in their quality. Some were passable and had enough good villi to make the necessary measurements but many were plagued with varied degrees of physical damage. This damage included: sheared villus tips, cracking of villi, and separation of enterocytes from the lamina propria. Additionally, the anatomical structure of the distal gut makes the consistent production of good quality slides uncertain, due to the presence of the annulospiral septa discussed earlier. The blocks were initially returned to PDS and personally re-cut by the head technician to absolutely rule out the possibility of a technical issue during sectioning and staining. The resulting slides were identical to the previous set, indicating that the observed damage almost certainly occurred prior to their delivery to PDS. Subsequent discussions with the head technician at PDS resulted in several suggestions to improve the quality of the slides.

Longitudinal sectioning, rather than cross sectioning, was suggested to avoid interference of the annulospiral septa. While longitudinal sections did provide an alternate viewpoint of the distal gut, the annulospiral septa are still a major anatomical feature of this region and are prominent on the slides. Due to the additional sample size required for longitudinal sectioning, which is extremely limited in smaller fish, it was decided not to continue with the method. Alternatively, taking duplicate cross sections of a single sample allows for reduced interference of the annulospiral septa without increasing the required sample size.

It was suggested by the PDS technician that perhaps the samples were not being properly fixed. He recommended either injection of the formalin fixative into a tied-off section of distal gut or that the gut section be opened lengthwise prior to fixing. After some limited testing and discussions amongst our research group it was determined that neither of these options were suitable. The limited availability of distal gut tissue makes tying-off a section impractical if not impossible. Opening a section lengthwise caused the sample to curl up and contort during fixation which was very undesirable for further processing. Despite these facts, based on the condition of the previous slides it was determined that the tissues, though damaged, appeared to be well fixed and properly stained, indicating that fixation was almost certainly not the causative issue.

Another possible cause of this type of damage is the physical handling of specimens. This type of damage can occur at any point prior to or during sampling and processing due to the delicate nature of the tissues. Up to this point, 1.5 cm samples were being fixed and subsequently rinsed and trimmed. Several variations of this procedure were tried, including the removal of the EtOH step. We were informed that EtOH drying is part of the procedure at PDS and is therefore not required prior to delivery. We also hypothesized that over-drying of the samples due to prolonged exposure to EtOH may result in the type of damage that we were seeing. As a result, four different procedures were tested in which the samples were either rinsed or not rinsed during trimming and were then placed into either 70% EtOH or fresh 10% neutral buffered formalin. It was expected that either the rinsing of digesta or the EtOH drying step would prove to have some kind of effect on the quality of the slides but no discernible differences were found between the four options.

The final suggestion from the PDS technician was to cut the samples, approximately 4 mm in length, directly into tissue cassettes. The cassettes were then placed directly into 10% neutral buffered formalin and delivered to PDS without further handling or processing of any kind. This procedure absolutely minimizes physical handling and entirely eliminates the trimming step previously required. It also concurrently increases the efficiency of fixation due to the reduced sample size and thickness. The only concession is that the tissue near the cut site would be compromised due to crushing from the scissors. As a result, several sections need to be discarded in order to reach undisturbed tissue during sectioning. This procedure was implemented as a final refinement to our protocol but the resulting slides, though slightly

improved, were still of insufficient quality to provide the necessary histological data, representative over the entire experiment.

The only conclusion that we can draw under these circumstances, in light of the extensive testing and refinements to our procedures, is that physical damage to the intestine occurred prior to sample collection. Due to the processes employed for capture of the fish, obtaining tank weights for performance evaluation, and method of euthanasia, I believe it is reasonable to make this conclusion. Tank weighbacks, which occurred one day prior to sample collections, were performed by net capture and transfer of the fish to an external container, then return to their tanks. This process is extremely stressful and has the potential to be physically traumatic for the fish. As one would expect, the fish struggle violently when they are caught in the net both during removal from and return to the tank. Particularly when the fish reach larger sizes, they are very strong and have the potential to incur injuries during this process. In addition to the potential for physical injury, it is not known what effects this extreme level of stress may have on the physiology of the animal.

The same process was employed, the following day, for sample collections. Three fish were selected from each tank and removed to an external container by net. They were then captured by net once again at the time of euthanasia. Due to the fact that the animals were not sedated, they had to be manually controlled to ensure efficient and humane delivery of euthanasia. Once again, the physical struggle of the fish in the net and manual control during euthanasia had the potential to damage the delicate tissues of the intestine. Despite every effort made to optimize the performance of these procedures, there is an inherent risk of physical injury to the animal. Additionally, the fish are certainly exposed to extremely high levels of stress during these final 24 hours prior to sample collection, which is of unknown consequence.

7.0 CONCLUSIONS

The overall results of this study indicate that inclusion of SBM and CM in aquaculture diets for rainbow trout should be limited. Negative impacts of both ingredients were evident in this experiment and careful consideration should be given to both the usage and inclusion rate of these ingredients. Conversely, dietary inclusion of PM was very well tolerated at inclusion levels up to 300 g kg^{-1}. These findings are in general agreement with previous studies including these ingredients, though mixed results exist in the literature likely due to varied experimental methodologies.

A major challenge of the current study was the high level of individual variation observed in mRNA transcript abundance. The factors influencing this variation were not clear; however, handling of fish to determine body weight the day prior to sample collection may have been a major contributor. Unfortunately, the level of variation observed warrants significant caution when interpreting the results. On the other hand, the positive correlations observed among genes associated with pro-inflammatory responses and concurrent negative correlations with anti-inflammatory genes adds confidence that transcript abundance levels are representative of expected biological responses.

Dietary inclusion of SBM was expected to be poorly tolerated at high rates. This ingredient has been consistently shown to have a detrimental impact on distal gut morphology, inflammation, and overall performance of teleost fish. Collins *et al*. (2012) found negative impacts on performance, particularly at high inclusion levels, with significant regressions for SGR, FCR, and PER. Despite the fact that this effect was not detected by the univariate GLM analysis, the results of the correlation analysis showed a clear association between SBM inclusion and the apparent upregulation of proinflammatory markers. The aqueous ethanol extraction process used to produce the SPC ingredient eliminated any observable negative impacts. With respect to ANFs, the compounds most closely related to inflammation, which have been identified as possible causative agents of soybean meal-induced enteritis, are the antigenic proteins glycinin and β-conglycinin (Rumsey *et al.*, 1994). These proteins are soluble in the alcohol fraction and are greatly reduced or eliminated during SPC production, which agrees with our results. Of the assayed ANFs, isoflavones are the only class that stand out for their content in SBM. While isoflavones have the potential for negative hormonal and nutritional impacts, their

effect on inflammation is not well characterized and the fact that they are only found in significant concentration in one ingredient renders any results of the correlation analysis null.

Mixed results exist in literature with regard to PM inclusion (Thiessen *et al.*, 2003; Drew *et al.*, 2005). Analysis of this experiment, perhaps surprisingly, did not reveal any negative impacts. High inclusion rates of PM could reasonably be expected to have a detrimental impact on teleost performance, due to its high starch content. Regardless, PM appears to have had an essentially neutral impact on performance and possibly influenced an upregulation of IL-10, which could be beneficial for reducing inflammation. Since PM was so well tolerated, processing of the ingredient to PPC would not be expected to be greatly beneficial, particularly since the starch content remains very high in relation to the other ingredients tested. Indeed, PPC was equally well tolerated and showed similar potential anti-inflammatory properties, in addition to a positive correlation with SGR not seen for PM. It is not clear what component(s) of the pea-based diets could be responsible for this apparently beneficial affect or why such a high starch content did not negatively impact performance. This is a somewhat unexpected result, since high-starch diets have been shown to negatively impact utilization of dietary energy, macronutrient digestibility, and growth of rainbow trout (Hilton *et al.*, 1983; Thiessen *et al.*, 2003).

Of the three plant-based proteins tested, CM was the least tolerated. Collins *et al*. (2012) found negative impacts on performance, with significant regressions for SGR, FCR, and PER. Though the univariate GLM analysis did not reveal any significant effects, correlation analysis showed a strong association with decreased ADFI and suggests that the mechanism of action for these effects is almost certainly not inflammatory. The aqueous extraction and dephytinization processes used to create the CPC did change the profile of the ingredient and appears to have improved its feeding value. With respect to ANFs, the best candidate for reducing both feed intake and performance, in the absence of an inflammatory response, is a palatability-mediated reduction in feed intake. Tannins, saponins, and glucosinolates present in CM should be considered individually and in combination for their contributions to reducing the palatability of the diet. Each of these components could have contributed to the responses observed and each was significantly reduced in the CPC ingredient.

In conclusion, we were able to differentiate the mechanisms by which SBM and CM inclusion negatively affect the health and performance of rainbow trout. We have identified that

pea-based ingredients may contain factor(s) that enhance performance, associated with stimulation of an anti-inflammatory response. With respect to PM and PPC, it is not clear why the high starch content of these ingredients did not have a measurable impact on performance. We have also highlighted that rainbow trout may be particularly sensitive to compounds such as tannins, isoflavones, and glucosinolates which affect the palatability of diets and can negatively impact both feed intake and performance. Furthermore, it has been confirmed that the production of purified protein concentrate ingredients has the potential to greatly reduce or eliminate negative impacts associated with ANFs. The additional cost associated with the sourcing and production of these ingredients must be considered when assessing their feasibility in relation to other viable options.

Overall, the results of this study demonstrate that not all plants are created equal. Recognition of the diversity in nutritional value, ANF content, and the potential for vastly differing impacts of various plant-based ingredients are essential. Feeding strategies based on the inclusion of beneficial components and investigation of the positive or negative interactions between complex dietary ingredients should be at the forefront. Increased knowledge of the mechanisms by which plant-based proteins affect performance, either positively or negatively, could aid in the design of complex diets that rely on multiple protein sources. The avoidance or enhancement of additive or even synergistic effects of individual ingredients affecting health and performance by the same mechanism would certainly be beneficial in this pursuit.

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